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NESIDIOBLASTOMA, THE ISLET TUMOR OF THE PANCREAS*

GEORGE F. LAIDLAW, M.D.

(From the Laboratory of Surgical Pathology, College of Physicians and Surgeons, Columbia University, and the Department of Surgery, Presbyterian Hospital, New York City)

This is a microscopic study of 9 "adenomas of the islets of Langerhans" removed surgically from 6 patients at the Presbyterian Hospital, New York City. It is a supplement to the report on these tumors published by Whipple and Frantz in 1935. The operations were performed for the relief of hypoglycemia with severe and long continued collateral symptoms of from 1 to 12 years duration. All patients recovered from the operation. In 5 patients the blood sugar rose and the collateral symptoms disappeared promptly. In Case 3, after removal of Tumor 3, there was no improvement. At a second operation, 1 month later, Tumor 4 was found and removed, together with 6 cm. of the tail of the pancreas, this time with prompt recovery.

The tumors were small, from 4 mm. to 2 cm. in diameter. In the tumors 2 cm. in diameter there was extensive fibrosis and calcification. In none of the tumors was there sufficient material for chemical or biological assay. The conclusions in this paper are based solely on histological staining, including specific staining of the cytoplasmic granules.

TUMOR PATTERNS

Structurally these tumors are nothing but gigantic islets of Langerhans, of which we have an excellent example in Tumor 1.

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Figure 1 shows how faithfully this tumor copies the structure of the normal islet. It reproduces the rich capillary network bordered by rows of columnar and cuboidal cells. As in normal islets, some of the capillaries have an endothelial lining, but many have none, the tumor cells seemingly being in direct contact with the blood. The tumor cells measure the same as cells of normal islets and are packed with fine granules which stain like the granules of the normal islet cells. As in normal islets, there is a minimum of fibrous connective tissue limited to a few strands along the capillaries and a delicate meshwork of argyrophil reticulin around the capillaries. Large areas of the tumor have no reticulin framework. Even with the highest magnifications and with a variety of stains, this tumor is indistinguishable from a normal islet except at the border where the adjoining acini are entangled and compressed in an incomplete fibrous capsule. The balance between tumor growth and blood supply is well maintained for all the cells appear healthy and there is no sign of necrosis or fatty degeneration.

Tumor 2, illustrated in Figure 2, is another gigantic islet of healthy cells. The figure shows the tendency of these tumors to exaggerate and repeat over and over some structural feature of a normal islet, in this instance the rosette arrangement of the cells around a capillary.

Figure 3 illustrates Tumors 5 and 6, removed at the same operation. This is an unusual pattern consisting of long ribbons of columnar cells with centrally placed nuclei. Each ribbon is a single row of cells lying between two capillaries. At many points no endothelial lining is visible and there is no fibrous connective tissue or argyrophil reticulin interposed between the tumor cells and the blood stream. The cells are packed with the specific islet cell granules. The cells of this tumor give an illusion of being abnormally large. By actual measurement they are quite uniformly the size of many normal islet cells.

Unusual as this pattern is, it is merely another instance of exaggeration and repetition of an ordinary islet figure. Short ribbons of this type occur in normal islets but the true prototype of the long ribbon is found in islet hypertrophy. MacCallum's picture of the ribbons in islet hypertrophy would serve as an excellent illustration of Tumors 5 and 6. Despite its resemblance to an embryonic structure, this ribbon pattern is not an embryonic or

undifferentiated form. Embryonic islets are more like the compact short ribbon type pictured in Figure 1.

As far as we know, no other islet tumor of the long ribbon type has been recorded, but the long ribbons of hypertrophy have been described by MacCallum, Cecil, and Weichselbaum and Stangl and regarded, mistakenly we believe, as an undifferentiated or regenerating form.

Hydropic Degeneration

Hydropic degeneration was not observed in any of our tumors. In the absence of exact knowledge we refrain from speculation on the possible relation to excess production of insulin.

Fibrosis

Pursuing our thought that the islet tumors are gigantic islets, we arrive at Tumors 3, 4, 5, 6, 8 and 9, all of which show more or less extensive fibrosis, hyaline degeneration and calcification. Here again the tumors are merely reproducing islet lesions on a grand scale; for non-tumoral islets are subject to precisely these changes — fibrosis, hyaline degeneration and calcification. The size of the tumors renders these lesions more impressive than when they occur in the tiny islets.

Fibrosis seems to be the common fate of these tumors. Of our 9 tumors, 6 present broad areas of fibrous connective tissue dotted with small groups of surviving tumor cells. In 1 of the 3 remaining tumors fibrosis is beginning in one sector. In most of the reports of islet tumors more or less extensive fibrosis has been recorded.

Figure 4 shows the fibrosis beginning in one sector of Tumor 1 as a thickening of the capillary wall studded with small blocks of collagen.

Figure 5 shows an advanced fibrosis, only a few tumor cells remaining; but these tumor cells are packed with the specific granules and they must have been active to judge by the prompt relief from the hypoglycemia after operative removal.

Hyaline Degeneration

In some of our fibrosed tumors much of the newly formed fibrous connective tissue has been converted into a clear glassy

substance which, in the negative outcome of amyloid and mucin reactions, we must be content to call hyalin. With Mallory's aniline blue collagen stain, or with its variants — Masson's trichrome and Heidenhain's azocarmine — the hyaline substance stains pale blue, much paler than the fibrous connective tissue. Many tumor cells contain similar pale blue patches in their cytoplasms. Studying these patches and the apparent conversion of entire cells to pale blue blocks, we are convinced that the tumor cells themselves undergo the hyaline as well as the fibrous connective tissue change, settling, in our own minds at least, the long-standing controversy as to whether the hyaline metamorphosis is restricted to the collagen or to the cytoplasm. It affects both.

This hyaline metamorphosis of the tumor cells has nothing to do with Bloom's D cells which stain with aniline blue.

Calcification

Three of our tumors are extensively calcified, which is not surprising considering the extent of the hyaline degeneration. As Mallory observes, hyaline material calcifies readily everywhere in the body.

Spontaneous Cure

On viewing the extensive destruction of tumor cells by fibrosis and hyaline metamorphosis, one surmises that this process might proceed to total obliteration of the tumor cells and a spontaneous cure (Bensley, O'Leary). Against this conclusion is the fact that tumors of several years duration and with extensive destruction of cells are still capable of producing hypoglycemia, as shown by the prompt relief of this condition after their removal.

The situation reminds one of chronic tuberculosis where, despite extensive healing by fibrosis and calcification, the healing process never quite overtakes the advance of the tuberculosis. Among islet tumors there is no authentic instance of spontaneous cure.

Nuclei

A word should be said about nuclei. Those pioneers in the study of islet cells, Lane and Bensley, described characteristic features of the nuclei of A and B cells and acinus cells, and their descriptions have been copied from one writer to another ever since with-

out adequate criticism. With the acid fuchsin-methyl green stain it is a simple matter to bring A cells, B cells and acinus cells into the same field for comparison. After a careful study of human and animal pancreas, and of the tumors, all fixed promptly in Zenker's or Bouin's fluid and properly stained, my conclusion is that there is nothing characteristic about the nuclei which distinguishes one of these cells from the other. When put to the practical test of diagnosis these meticulous nuclear distinctions break down, as they did with one experienced cytologist and student of the pancreas (O'Leary), who studied the 5 St. Louis tumors under the most favorable conditions — immediate fixation and expert staining — and concluded that "these characteristics are hardly sufficient to distinguish one type of cell from another." We agree with him.

Specific Granules

Islet cells and the cells of the islet tumors differ from most cells in the body by being packed with fine granules. These are probably secretion granules (O'Leary). They are not artefacts for they are visible in fresh islets (Laguesse, Bensley, Covell, O'Leary). Laguesse stained these granules with safranine; Lane with gentian violet and orange G; Martin with ethyl violet and orange G; Bowie with ethyl violet and Biebrich scarlet; and Bensley with a variety of methods, including acid fuchsin and methyl green. These stains were devised for the pancreas in the lower animals. In our hands, when applied to human pancreas and human tumors, these stains with one exception proved to be exasperatingly capricious. The exception was Bensley's acid fuchsin-methyl green, which we found to be simple, accurate and constant in all kinds of islets, normal and pathological, and in islet tumors.

If normal pancreas is fixed in Zenker's fluid and paraffin sections are stained with acid fuchsin and differentiated in methyl green, the acinus cells are green with green nuclei, the zymogen granules red, and the basal filaments and mitochondria red. In contrast with the green acinus cells the islet cells are packed with fine red granules. With a slight modification of the technique the granules of Lane's A cells hold the red, while the granules of the B cells turn purple. The tumor cells react to this stain exactly like islet cells. In most of the tumor cells the granules take the

purple color of B cells with here and there a red A cell. It should be noted that to get good differentiation of islet cells, human pancreas requires stronger and longer staining than the pancreas in the lower animals. In the tumors we have not found Bensley's granule-free C cells and we agree with O'Leary in failing to find any of Bloom's D cells that stain with aniline blue. D cells are supposedly brought out best by fixation in Helly's fluid (Zenker-formol), in which some of our tumor tissue was fixed.

Origin of the Tumors

In the pancreas, the duct epithelium is the source of all growth and repair (Bensley, Norbert, Grauer). In the embryo, epithelial buds from the duodenum grow toward the spleen as branching pancreatic ducts. This duct epithelium is totipotent, as Bensley calls it, for at one point it differentiates into acinus cells, at another point into islet cells, and at still other points it pushes forward as branching ducts. The duct epithelium retains this totipotency throughout life, as shown by the remarkable instances of regeneration of the pancreas from the ducts, reproducing the pancreatic structure complete, with acini, islets and ducts, amounting in several instances to regeneration of the entire pancreas of the adult rabbit (Grauer). Once differentiated out of the duct epithelium the islets grow by proliferation of their own cells (Bensley).

Curiously enough a stimulus that calls forth the duct-building and islet-building potency of the pancreas, while leaving the acinus-building potency in abeyance, is known. After ligature of the ducts both acini and islets degenerate and disappear, or nearly disappear (Bensley). If ligation is continued the islets regenerate from the ducts but the acini do not. If, on the other hand, the ligature is removed and free drainage of the duct system reestablished, the acini regenerate as well (Bensley, Harvey, Grauer).

The islet tumors may be regarded as a reaction of the duct epithelium to a stimulus that has called forth its duct-building and islet-building potencies, leaving the acinus-building potency in abeyance. Figure 6 from Tumor 3 shows the process in full swing. Throughout the tumor, ducts are so numerous that most of them must be accepted as newly formed. In the center of the figure a duct is seen, easily recognizable by the terminal bars or

"Schlussleisten" which fill the chinks between the epithelium. The epithelium of the duct is continuous with a group of tumor cells, as if the tumor cells were differentiating out of the duct epithelium.

Similar abundance of ducts and continuity of duct epithelium with tumor cells is found in every one of our tumors. O'Leary observed similar figures in 4 out of 5 of the St. Louis tumors, and interprets them in the same way. O'Leary observes justly that the mitotic figures in some of the tumors show that once differentiated out of the duct epithelium the tumor cells possess the power of independent proliferation.

In the normal pancreas such continuity of duct epithelium and islet cells is a matter of common observation (Laguesse, Bensley, and others). It is even asserted, on the evidence of serial sections, that the islets of the adult pancreas never lose their original continuity with the duct epithelium. The formation of the islet tumors, then, is merely an exaggeration of a normal procedure — differentiation of islet cells out of duct epithelium and subsequent independent growth.

Concerning mitoses and infiltration, we quote from Whipple and Frantz: "We have classified these eight tumors as adenomata, for the present at least, and only in the fifth, sixth, and eighth have we seen any evidence of what might be considered an infiltrating tendency. Marked variation in the size and shape of cells, mitotic figures in any appreciable number and blood vessel invasion are nowhere present."

NESIDIOBLASTOMA

There is need for a short and accurate name for these tumors. Adenoma of the islets of Langerhans is long and cumbersome. Adenoma itself is vague, for we have already two kinds of adenoma, the benign epithelial tumor and lymphadenoma, quite different from each other. To add still another adenoma, an endocrine variety, merely adds to the confusion. We have followed current custom of suffixing "oma" to the Greek name of the cells of origin of the tumor. Selecting *νησίδιον* as the Greek word for islet, the cells that differentiate out of the duct epithelium to build islets may be called nesidioblasts — islet builders. When these islet builders, or nesidioblasts, form tumors, the tumor is a nesidioblastoma. The name has another application. In contrast with

the concentration of excess islet tissue in a tumor there is some evidence pointing to a diffuse or disseminated proliferation of islet cells as a possible cause of hypoglycemia. Such a diffuse proliferation of nesidioblasts would be a nesidioblastosis.

Summary

Microscopically the chief feature of most of the tumors is their exact duplication of the pattern of normal islets. They also resemble islet hypertrophies in their tendency to exaggerate some features of the normal islet pattern. Just as the tumors duplicate the structure of normal and hypertrophied islets, so they are subject to the same pathological vicissitudes such as fibrosis, hyaline degeneration and calcification. The origin of the tumor cells is indicated by the abundance of figures showing the epithelial lining of the duct continuous with a group of tumor cells.

The origin of the name "nesidioblastoma" is explained.

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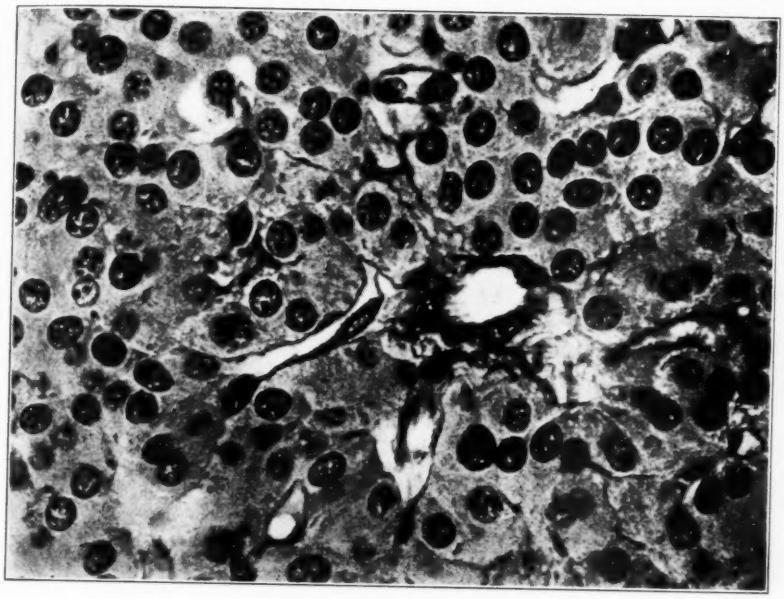
DESCRIPTION OF PLATES

PLATE 27

FIG. 1. Tumor 1. The photomicrograph shows how the tumor faithfully reproduces the structure of the normal islet with its rich capillary network bordered by rows of columnar and cuboidal cells. Azocarmine stain. × 730.

FIG. 2. Tumor 2. In this tumor there is a rosette arrangement of the cells around capillaries. This reproduces one feature of normal islets. Azocarmine stain. × 730.



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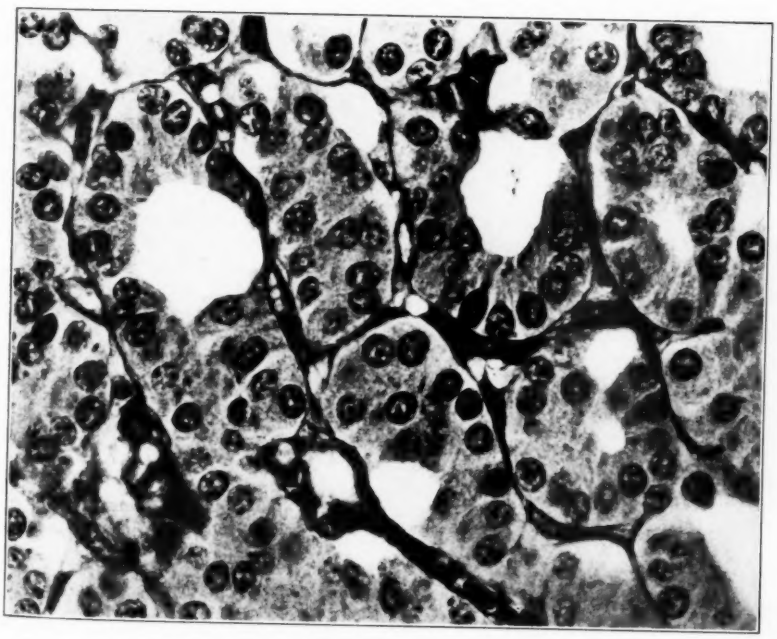

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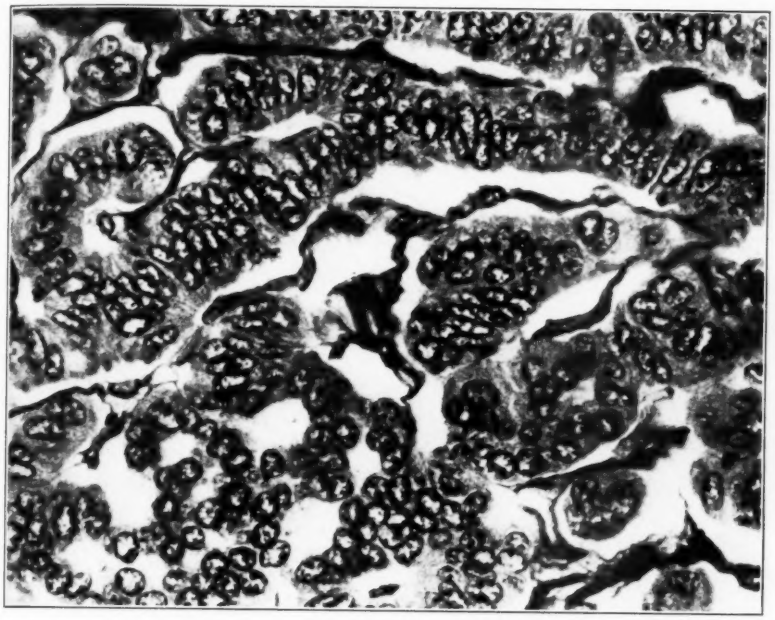
PLATE 28

FIG. 3. The photomicrograph shows the pattern of Tumors 5 and 6; long ribbons of columnar cells with centrally placed nuclei, each ribbon a single row of cells lying between capillaries. Azocarmine stain. × 730.

FIG. 4. This section of Tumor 4 shows fibrosis beginning as thickening of the capillary walls studded with small blocks of collagen. At A is shown the lumen of a capillary and at B a small block of collagen in its wall. Other collagen masses may be distinguished by the absence of granules. Masson's aniline blue-acid fuchsin-ponceau stain. × 730.



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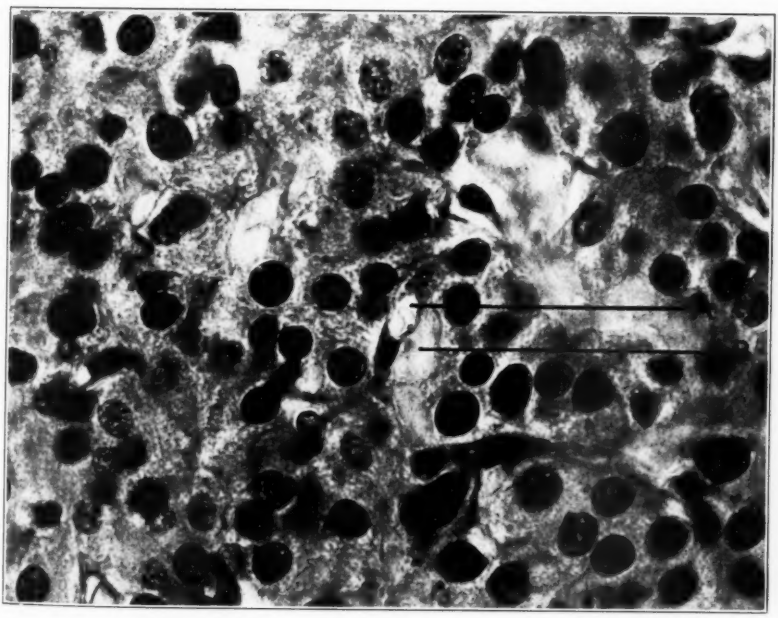

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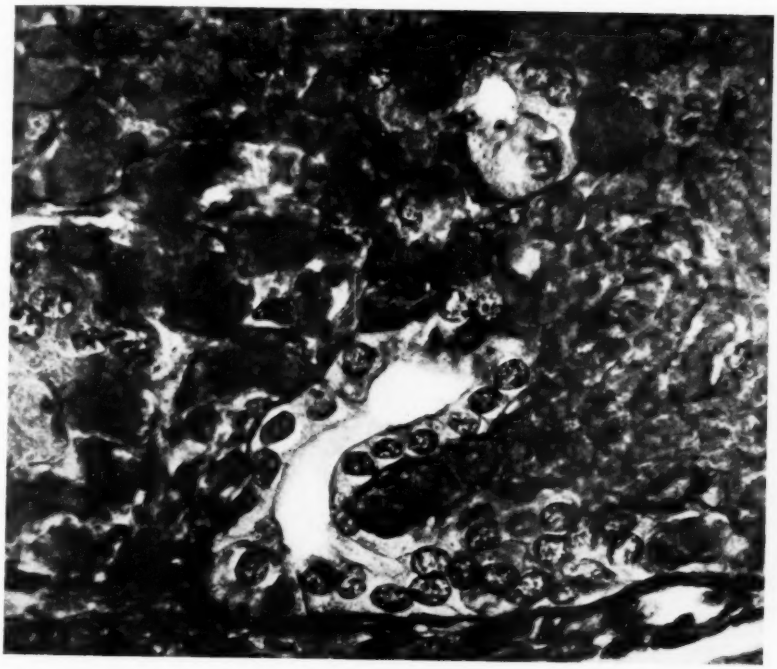
PLATE 29

FIG. 5. Tumor 3. An example of advanced fibrosis; only a few tumor cells remain but they are apparently active, judging by the presence of granules and the prompt relief of hypoglycemia after their surgical removal. Azocarmine stain. × 730.

FIG. 6. Tumor 3. This shows the epithelium of the duct in continuity with a group of tumor cells, as though the tumor cells were differentiating out of the duct epithelium. The Schlussleisten are shown as black dashes between the cells lining the lumen and radiating from it. Masson's aniline blue-acid fuchsin-ponceau stain. × 1600.



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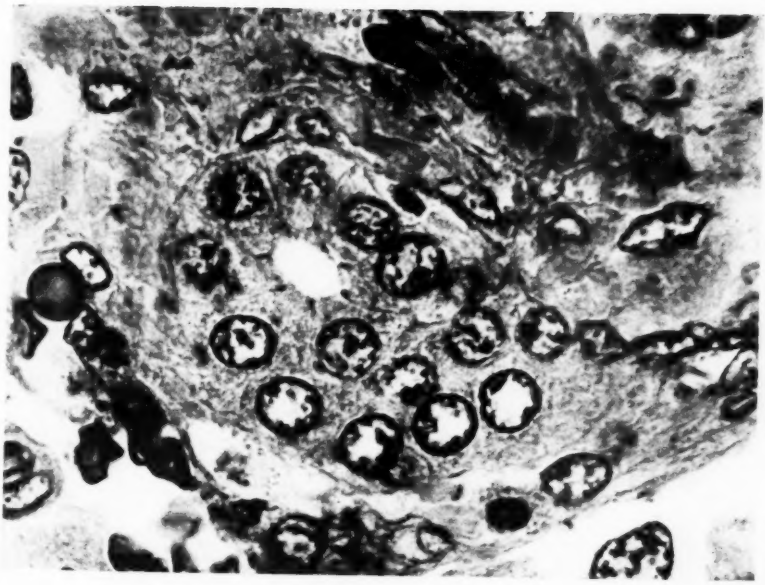

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DEVELOPMENTAL DEFECTS AT THE FORAMEN OVALE *

BRADLEY M. PATTEN, PH.D.

(From the Department of Anatomy, University of Michigan Medical School, Ann Arbor, Mich.)

Many individual instances of patent foramen ovale are already on record in the literature. These cases, however, do not seem to have been studied as a group, either with a view to differentiating the types of malformation encountered in this location, or ascertaining for the different kinds of defects the possible range of their variation in extent. A long-standing interest in the normal and defective development of the heart has, through the generous cooperation of colleagues, brought to me for study more material of this type than one person would ordinarily encounter. Recently a leave of absence afforded the further opportunity of studying the specimens accumulated in a group of pathological institutes with records covering a total of over 500,000 autopsies. Naturally not all the congenitally defective hearts from these autopsies had been preserved, but the extensiveness and variety of the material available was exceptional. Using drawings made directly from my own or museum specimens as a basis, and supplementing this material from a study of the literature, I have attempted to assemble a brief, but freely illustrated, survey of the defects that may be encountered at the foramen ovale. Being not a clinician but an embryologist, I have approached the subject from a morphological standpoint. It is hoped, however, that the material may prove a useful foundation for those interested in attacking the clinical problems associated with such defects.

LITERATURE

Publications dealing with failure of the foramen ovale to close have been appearing for more than three centuries. Many of the papers are so old that their viewpoint has become almost unintelligible to us of today. Botalli in 1565, for example, seized on cases exhibiting an open foramen ovale as offering an improvement on Galen's idea that the blood entered the left side of the heart from the right by way of spaces between the trabeculae of the interventricular septum (Dalton, 1884, p. 137). The weight

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of Botalli's name behind this erroneous conception delayed for many years the acceptance of Servetus' contention that the passage of blood from right to left "does not take place through the median wall of the heart as commonly believed; but, by a grand defice, the refined blood is driven from the right ventricle of the heart, in a long course through the lungs." The language in which Servetus elaborated his ideas well indicates the curious mixture of keen observation and dogma that pervaded the work of this period. "By the lungs it (the blood) is prepared, assuming a bright color. It is mingled with the inspired air and purged of its fulginous matter by expiration and so at length the left ventricle of the heart attracts by its diastole the whole mixture, a suitable . . . material that . . . may become vital spirit." (Translation from Dalton, 1884, p. 115.)

Unfortunately the old papers are by no means the only ones in the literature that throw little light on the subject. Many comparatively recent articles are but superficial descriptions of isolated cases. An idea of the frequency with which papers based on 1 or 2 cases appear in the literature may be gathered from the fact that in 205 references cited by Poynter (1919) only 225 cases are involved. Many of these were merely clinical diagnoses of "open foramen ovale" with no confirmation by autopsy. Among the enormous number of papers on the subject disappointingly few contain both a good clinical history of the case and an adequate record of the autopsy findings.

Viewing the literature as a whole there seem to have been three factors primarily responsible for the often contradictory and unsatisfactory information it contains. First is the deep rooted tradition that the foramen ovale closes immediately following birth. Thus, in the absence of other findings accounting for death, an open foramen ovale in a young infant is frequently unjustly accused. This has led to much misapprehension as to both the frequency of occurrence, and the functional significance, of an unclosed foramen ovale during the neonatal period. There has long been ample evidence that the foramen ovale is not closed immediately after birth, but that its closure is a gradual process spreading over most of the first year (Aleksïeyeff, 1901; Alvarenga, 1869; Elsässer, 1852; Hinze, 1893; Patten, 1930, 1931; Scammon and Norris, 1918). Familiarity with this fact would have eliminated

from the literature many papers describing as instances of "abnormal patency of the foramen ovale" conditions perfectly normal for the age at which they were observed. For example, a paper published comparatively recently in a well known medical journal is based on the heart of an infant that lived but 6 hours after birth. Death was attributed to an open foramen ovale and an unclosed ductus arteriosus!

A second cause of confusion commonly encountered in the literature is the failure to distinguish between conditions in which the foramen ovale is adequately covered by a valve which is not completely adherent to the septum, and conditions in which a structural defect of the valve or the septum makes it impossible for the foramen to be functionally closed. Incomplete adhesion of the valvula to the septum, with a resulting "probe-patency," is so common that it must be regarded as a variant of the normal rather than as an abnormality. The combined figures of ten different observers compiled from over 4000 autopsies in which this condition was an object of special attention show that probe-patency exists in one out of every four or five adult hearts (see Table I). As long as the valvula foraminis ovalis adequately overlaps the limbus fossae ovalis, probe-patency appears to be no functional handicap to an otherwise normal individual. The inclusion in the literature of a large number of cases where the "defect at the foramen ovale" was mere probe-patency has led to the impression that functionally significant defects in this region are much more common than is actually the case.

Still a third underlying difficulty in arriving at any clear interpretation of the significance of defects at the foramen ovale is one that seems inherent in the entire subject of congenital defects of the heart. There appears to have been a sort of collector's instinct obsessing contributors to this field. The more bizarre and complicated the case, the more interest it appears to arouse. From either the practical or the scientific standpoint this is unfortunate. The clinical picture especially is most confusing when several defects co-exist in the same heart. The only hope of arriving at any sound interpretation of such cases lies in better understanding of the developmental conditions responsible for, and the clinical manifestations of, uncomplicated cases of specific defects in which the major characteristics of the condition stand out unequivocally.

To attempt to give a systematic survey of all the articles in a field where such a large proportion of the material is either antiquated or uncritical would not be profitable. In the course of preparing this paper about 3000 references on congenital defects of the heart were culled. Some 300 of these purported to deal with an open foramen ovale. Even this burdensome list undoubtedly fails to constitute a complete bibliography, for the literature is scattered among journals dealing with clinical medicine, pathology, physiology, anatomy, embryology, and even general biology. It has, therefore, seemed wiser to dismiss the literature as a whole with the foregoing general comments and deal only with a relatively few selected references in connection with matters on which they were found helpful.

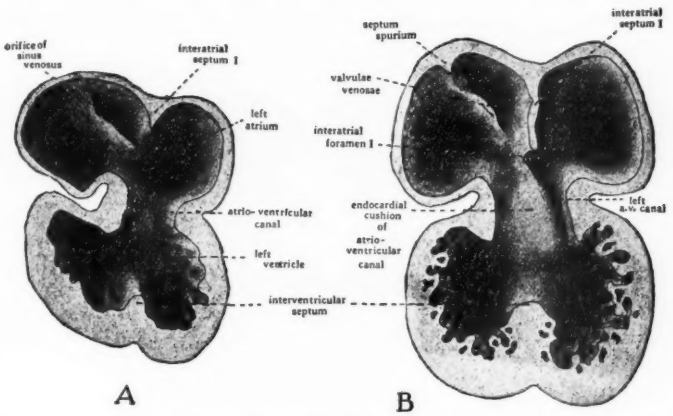
The Development of the Interatrial Septal System

The growth processes leading toward the establishment of conditions as they appear in the heart of a newborn infant and the changes in the heart following birth are fairly well covered in the embryological literature (Born, 1889; Keibel and Mall, 1910; Mall, 1912; Odgers, 1935; Patten, Sommerfield and Paff, 1929; Tandler, 1912 and 1913; Waterston, 1918). Much of this information, however, is so widely scattered and so uncorrelated that it is not readily utilizable by those working in other fields. For this reason, and also for the sake of emphasizing certain points especially pertinent to an understanding of the defective conditions under discussion, the following brief summary of the normal prenatal and postnatal development of the interatrial septa is given.

In the separation of the primitive common atrium into right and left chambers two septa are directly involved. These, on the basis of their sequential appearance, are commonly called septum primum and septum secundum. The partitioning process starts in very young embryos, indications of the formation of septum primum being recognizable as early as the 5th week * of development. Starting as a crescentic ridge on the dorsocephalic part of the atrial well, septum primum grows toward the atrioventricular canal (Text-Figs. 1, A and 4, A).

* Ages as here given are approximate fertilization ages, for "menstrual age" add 14 days.

At about the same time that septum primum is making its appearance, the first indications of the impending division of the original common atrioventricular canal into a right and a left channel become evident. Two local thickenings, one dorsally, the other ventrally located, appear in the walls of the canal. These thickenings are the so-called endocardial cushions of the atrioventricular



TEXT-FIG. 1. Semischematic drawings of the interior of the heart to show the initial steps in its partitioning. (From Embryology, Patten, B. M., courtesy P. Blakiston's Son and Co.)

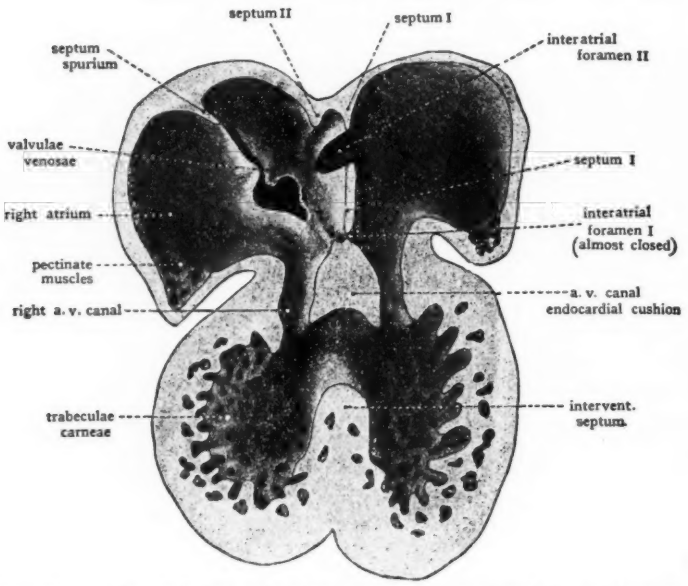
A. The cardiac septa are represented at the stage reached in human embryos early in the 5th week of development. Note especially the primary relations of interatrial septum primum. Based on original reconstructions of the heart of a 3.7 mm. pig embryo, and on Tandler's reconstructions of corresponding stages of the human heart.

B. The cardiac septa as they appear in human embryos of the 6th week. Note the restriction of interatrial foramen primum by the growth of interatrial septum primum. Based on original reconstructions of the heart of a 6 mm. pig embryo, on Born's reconstructions of the rabbit heart, and Tandler's reconstructions of corresponding stages of the human heart.

canal. Each cushion consists of a plastic mass of embryonal connective tissue, of the type characteristically appearing in the developing heart at points where septa will fuse, or where elaborate connective tissue structures such as the cardiac valves are destined to be moulded. During the 6th week of development the dorsal and ventral cushions are brought into contact with each other by their own growth and fuse to form a common mass dividing the

atrioventricular canal (*cf.* Text-Figs. 1 and 2, and Text-Fig. 4, C and D).

Between the concave margin of septum primum and the growing atrioventricular canal cushions is a progressively diminishing opening known as the interatrial foramen primum, or ostium primum



TEXT-FIG. 2. Semischematic drawing of the interior of the heart to show the start of interatrial septum secundum and the appearance of interatrial foramen secundum in septum primum. Based on original reconstructions of the heart of a 9.4 mm. pig embryo and on Tandler's reconstructions of the heart of human embryos of the 7th week. (From Embryology, Patten, B. M., courtesy P. Blakiston's Son & Co.)

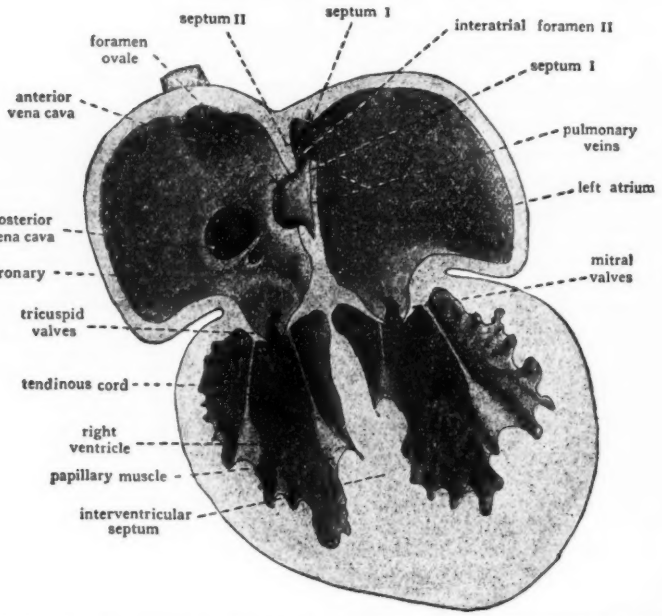
(Text-Fig. 1, B and 4, B). About when it seems as if the closure of the ostium primum would shut off the left atrium from the right (Text-Fig. 4, C) a secondary opening develops in septum primum near its origin from the dorsocephalic atrial wall. This new aperture first appears as multiple small perforations which soon coalesce to form a single opening known as the interatrial foramen secundum, or more briefly, as ostium secundum (Text-Fig. 2, and Text-Fig. 4, C and D).

The appearance of a second interatrial communication just as

the initial one is closing is of fundamental functional significance. In early embryonic life, when the lungs are as yet undeveloped, the left atrium lacks any considerable direct intake of its own. The constant presence of an interatrial communication makes it possible for the left atrium to receive without interruption a contribution from the blood entering the right atrium. More than the atrial part of the heart is involved in this matter of balanced atrial intakes for, as we have seen, the atrioventricular canal is divided by the 10 mm. stage, and at about the 15–17 mm. stage the interventricular septum separates the right and left ventricles from each other. After these partitions are completed, if the atrial intakes were unbalanced the ventricular intakes would inevitably be correspondingly disturbed. That this is a matter of more than theoretical importance is clearly shown by the conspicuously defective development of the left side of the heart which is encountered when, as occasionally happens, abnormal development prematurely closes or markedly narrows the interatrial communication of the fetal heart. (A case of this type is presented later in this paper, see Figs. 17, 18 and 19.)

About the time the secondary interatrial opening is formed in septum primum, another septum begins to develop. The second septum is usually first readily recognized in embryos of about 12 mm. (end of 6th week), although occasionally its beginnings may be made out somewhat earlier. Like septum primum, septum secundum is crescentic in shape, but the open part of the crescent is directed more dorsally — toward the sinus inlet rather than toward the atrioventricular canal as was the case with septum primum. In reconstructions of the developing heart septum secundum can be seen lying just to the right of septum primum (Text-Fig. 2). Its cephalodorsal limb extends along the dorsal wall of the atrium with its tip lying in close association with the left valve of the sinus venosus. The ventrocaudal limb of septum II extends along the ventral walls of the atrium, sweeps caudally and merges with the atrioventricular canal cushion just to the right of the place where septum primum fuses with the canal cushion to obliterate the primary interatrial foramen (ostium I) (Text-Fig. 2 and Text-Fig. 4, D). The extreme tip of the ventrocaudal limb of the septum extends to meet the tip of the cephalodorsal limb at the base of the left valve of the sinus venosus (Text-Fig. 2).

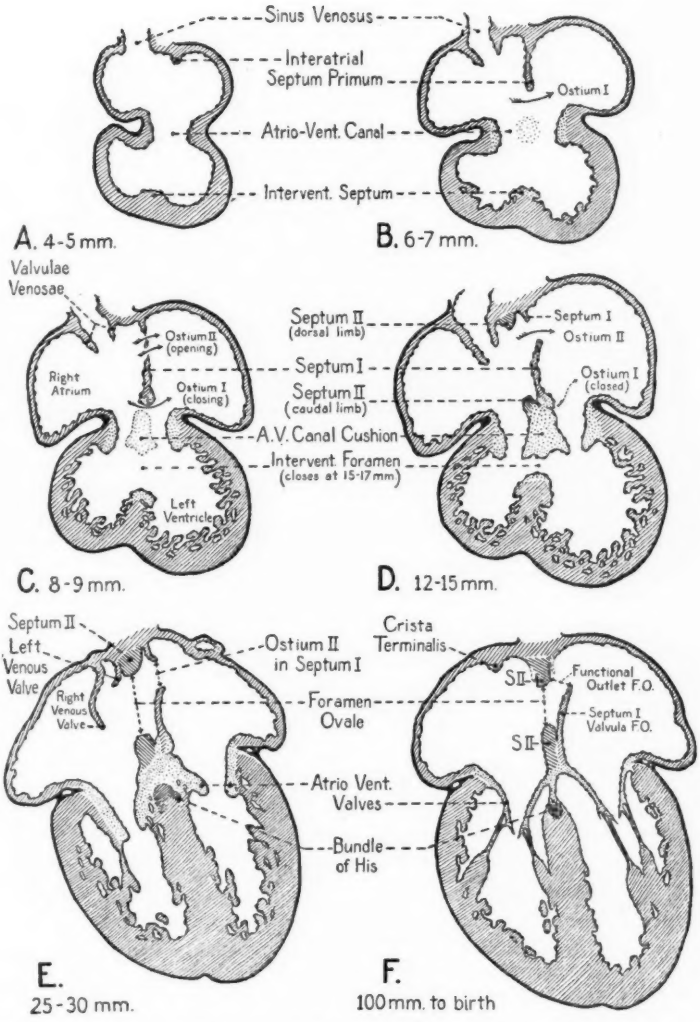
As septum secundum grows, its concave margin for a time cuts progressively farther into the atrial lumen; but septum II is not destined to become a complete partition. Its extension gradually ceases, leaving a characteristic oval aperture which is the foramen ovale (Text-Fig. 3 and Text-Fig. 4, E, F). The margin of septum



TEXT-FIG. 3. Schematic drawing to show the interrelations of septum primum and septum secundum during the latter part of fetal life. Note especially the way in which the lower part of septum primum is situated so it acts as a one-way valve at the oval foramen in septum secundum. (From Embryology, Patten, B. M., courtesy P. Blakiston's Son & Co.)

secundum thus constitutes what in adult anatomy is called the limbus or annulus fossae ovalis.

The relations of septum primum to the oval foramen persisting in septum secundum are of vital importance. The secondary opening in septum primum is formed so near the cephalic wall of the atrium that the unresorbed lower part of septum primum lies as a loose flap covering, on its left atrial side, the oval opening in septum secundum (Text-Fig. 3 and Text-Fig. 4, F). In this position it acts as one-way valve, permitting the filling of the left atrium



TEXT-FIG. 4. Sectional plans of the embryonic heart in the frontal plane, showing extent of growth of the various cardiac septa at several stages of development. These diagrams give specifically for the human embryo a more precise picture of the rate of progress of partitioning than do the schematic drawings of Text-Figs. 1–3.

Stippled areas in the diagrams indicate the distribution of endocardial connective tissue, muscle is shown in diagonal hatching, and the epicardium in solid black. The lightly stippled areas in the atrioventricular canal in B and C indicate the location of the dorsal and ventral endocardial cushions of the atrioventricular canal before they have grown sufficiently to fuse with each other in the plane of the diagram.

from the right but effectively shutting off return flow. In the fully formed fetal heart this flap is commonly known as the valvula foraminis ovalis rather than by its embryological name, septum primum.

Thus during intrauterine life we find a succession of three morphologically distinct interatrial communications, the first below septum primum, the second in septum primum, and the final one in septum secundum. This permits the left atrium, throughout fetal life, to receive a contribution of blood from the right atrium by a transseptal flow which compensates for the relatively small amount of blood entering the left atrium by way of the pulmonary circuit, and maintains an approximate balance of intake into the right and left sides of the heart. The amount of this compensatory interatrial flow changes in relative volume at different ages. Early in development, before the lungs have been formed, the flow from the right atrium through the interatrial ostium primum constitutes the entire intake of the left atrium. After ostium primum is closed and while the lungs are but little developed, flow through the interatrial ostium secundum must still be the major part of the blood entering the left atrium. During the latter part of fetal life the foramen ovale in septum secundum becomes the transseptal route. As the pulmonary circulation increases in volume, a progressively smaller proportion of the left atrial intake appears to come by way of the foramen ovale and a progressively larger amount from the vessels of the growing lungs. At the time of birth, on the basis of orifice measurements, somewhat more than half the blood entering the left side of the heart appears to come from the lungs and less than half from the right atrium by way of the foramen ovale (Patten, 1930).

The progressively diminishing transatrial flow and the progressive increase in the volume of the pulmonary circulation during the latter part of fetal life seem to have been largely overlooked. Unfortunately, no one has as yet solved the difficult problem of obtaining from living embryos pressure and volume determinations such as would permit a quantitatively accurate evaluation of the situation. But, if we may judge anything from the size of the vessels concerned, the volume of the pulmonary circulation of a term fetus is far from the negligible quantity commonly assumed. On the contrary, it is probably already sufficient to take care of

gaseous interchange as soon as the lungs are ventilated, for the pulmonary arteries of a term fetus are of approximately the same size as its umbilical arteries, and the total cross sectional area of the pulmonary veins is approximately equivalent to the cross section of the umbilical vein (Patten and Toulmin, 1930). If we recognize the fact that pulmonary vessels as large as the umbilical vessels can carry a volume of blood sufficient for gaseous interchange we are at once relieved of the necessity of postulating the traditional revolutionary changes in circulation at the moment of birth. Postnatal circulatory changes can then be interpreted on the basis of gradual readjustments which are more in harmony with what we know of other processes of change in living organisms.

Following birth, the lumen of the ductus arteriosus is gradually occluded by an overgrowth of its intimal tissue. The histological picture presented is somewhat suggestive of the changes seen in endarteritis obliterans. This process in the wall of the ductus is as characteristic and regular a feature of the development of the circulatory system as the formation of the cardiac septa. Its earliest phases begin to be recognizable in the fetus as the time of birth approaches, and after birth continue at an accelerated rate to terminate in complete occlusion of the lumen of the ductus about 6 to 8 weeks after birth. This progressive closure of the ductus arteriosus reduces the shunt from the pulmonary circuit to the aorta and, acting together with the newly assumed respiratory activity of the lungs themselves, gradually raises the pulmonary circulation to full functional level. Barcroft (personal communication) is inclined to believe on the basis of recent experiments that there is also contraction of the smooth muscle in the wall of the ductus following birth. If this proves to be the case it would mean that the increase in pulmonary circulation is accelerated by a physiological mechanism which begins to act more promptly than the mechanism of morphological closure. It is difficult to conceive of such muscular action closing the ductus immediately and completely and being effectively maintained during the 6 to 8 weeks occupied by morphological closure. Nevertheless such a vasoconstriction might well reduce flow through the ductus sufficiently to accelerate the increase in blood flow to the lungs and to facilitate the ultimate closure of the ductus arteriosus by the growth of its own intimal tissue.

The results of increased pulmonary circulation with the concomitant increase in the direct intake of the left atrium are manifested secondarily at the foramen ovale. Even before birth — in the latter part of fetal life as the lungs attained considerable development — we noted that a reduction in transseptal flow was beginning to be evidenced. Following birth, as the pulmonary return increases still more, compensatory blood flow from the right atrium to the left decreases correspondingly. This is indicated anatomically by a progressive reduction in the looseness of the valvula foraminis ovalis and the consequent diminution of the interatrial communication to a progressively narrower slit between the valvula and the septum. This first phase in the closure of the foramen ovale occupies approximately the 1st postnatal month, during which time the pulmonary return is mounting toward equivalence with the right atrial intake. When this equalization has occurred, the compensating one-way valve at the foramen ovale falls into disuse. Although a probe can still be passed freely behind the valvula, the foramen ovale may be regarded as functionally closed when this new intracardiac balance has been attained.

Then follows a period of 6 to 8 months in which the connective tissue of the valvula increases from 600 to 700 per cent (Patten, 1931). Probe-patency still persists but the size of the slit through which a probe may be passed progressively diminishes and the resistance to its passage increases with the increase in the thickness of the valvula. This second phase in the closure of the foramen ovale with its characteristic histological alteration is essentially the conversion of an originally movable, flap-like valve into a fixed septal structure.

Finally, coming leisurely in the wake of functional abandonment and as a culmination of the period of connective tissue proliferation, is the adhesion of the valvula to become an integral part of the interatrial septum. There is great individual variability in the age at which this final step in the closure of the foramen ovale occurs. A usual range, rather than a specific time of final anatomical closure, is all that can be specified. Substantiated cases of the fibrous adhesion of the valvula to the septum becoming complete under 3 months are exceedingly rare. The usual time of complete anatomical closure appears to be not earlier than the last 3rd of the

1st year after birth, and is frequently much later (Patten, 1931).

In 20 to 25 per cent of adult individuals the fibrous adhesion is never entirely completed (Table I). Provided the valvula amply

TABLE I

Records as to Completeness of Closure of Foramen Ovale, from a Large Series of Individuals Beyond Childhood

Observer	No. of cases examined	Not completely closed
Adami–Abbott, 1915	1374 (adults)	199
Bizot, 1837	155 (mostly adults)	44
Brit. Anat. Soc., 1897 (Parsons and Keith)	316 (all above 10 yrs.)	76
Fawcett and Blachford, 1901	306 (all over 6 yrs.)	96
Hinze, 1893	359 (all over 20 yrs.)	82
Ogle, 1857	62 (adults)	13
Rostan, 1884, and Zahn, 1889	711 (661 over 20 years)	139
Seib, 1934	500 (all over 20 yrs.)	85
Wallmann, 1859	300 (291 over 20 yrs.)	130
Totals	4083	864

Foramen ovale not completely closed in 21.2 per cent of cases.

The exact percentage incidence of unclosed foramen ovale obtained by compiling such data naturally varies with the length of the series of cases and the criteria used in selecting acceptable data. In a previously compiled table for about 4000 cases, "mostly adult" but not rigidly selected for age, the per cent obtained was 24.6 (Patten, 1931). In a compilation of 2648 cases in which all cases under 20 years were excluded, Seib (1934) arrived at a figure of 23.1 per cent. The present table showing 21.2 per cent differs from my own previous one in the substitution of Seib's new series of 500 cases in which the ages were all known to be above 20 years, for the 500 cases of Klob in which no account was taken of ages, and which one suspects from the 45 per cent of non-closures must have included many very young individuals. The present table differs from Seib's in containing a considerably greater number of cases because of less rigid age selection. The point to be emphasized is the essential consistency of these three tabulations, rather than their minor variations. For all practical purposes we may say that in individuals beyond childhood we may expect 1 case out of every 4 or 5 to show an incompletely closed (*i.e.* "probe-patent") foramen ovale.

overlaps the foramen ovale such failures of complete adhesion appear to be no functional handicap to an otherwise normal individual. Because of this fact and the frequency with which they occur, these cases may well be regarded as variations of the normal rather than as abnormalities. Such an attitude, however, must be tempered by the realization that in the event of disturbances in the pulmonary circuit sufficiently severe to unbalance intra-atrial pressures, an area of incomplete adhesion may again become a path for transseptal flow. The interesting experimental work of Gross (1934), in which he observed the behavior of interatrial septa obtained at autopsy and clamped between artificial atria in which the pressures could be varied at will, clearly demonstrates that this is more than a mere theoretical possibility.

With this brief sketch of prenatal and neonatal conditions as a background we may turn to a consideration of the various types of developmental defects which may manifest themselves at the foramen ovale.

Congenital Defects at the Foramen Ovale

Congenital defects of the heart are commonly attributed to one of two alleged causes: to "developmental arrests" which are said to leave some essential cardiac structure in an "underdeveloped" condition characteristic of a phase of its formation during embryonic life; or to the damaging effects on local growth of some inflammatory process that becomes established during fetal life. Neither of these factors adequately accounts for the wide variety of congenital defects encountered either at the foramen ovale or other locations in the heart.

As far as the evidence from any material that I have seen is concerned, pathological lesions rarely, if ever, appear to play a part in the primary causation of a developmental defect at the site of the lesion. Inflammatory reactions resembling those caused in the adult by endocarditis undoubtedly do occur occasionally at the site of congenital defects. There is, however, no reason to believe that such a process is the cause of the congenital defect. On the contrary, the lack of any semblance of constancy in the association of such lesions with developmental defects in general points very strongly to the conclusion that the association, when it does occur, is fortuitous. Possibly a defect of such a nature that it constitutes

a point of local stress, as for example pulmonary stenosis, may furnish a site of predilection for an inflammatory process, once the causative agent has become established in the fetal blood stream. That a localized inflammatory process causes a developmental defect at the site of the lesion appears to be unsupported by any valid evidence.

While local pathological lesions may be discounted, or even dismissed altogether, as direct causative agents, congenital defects which might be interpreted as developmental arrests unquestionably occur. If, for example, septum secundum does not grow to the usual extent, the orifice to be occluded by the valvula foraminis ovalis remains abnormally large and, therefore, may be inadequately guarded by a valvula which is itself perfectly normal (Fig. 5). In such a case we might properly employ the expression "developmental arrest," for growth progressing along its normal course has fallen short of completion.

There are, however, defects at the foramen ovale that are in no sense the result of the cessation of a growth process short of its usual culmination. If, for example, the normal process of resorption which is concerned in the establishment of the secondary opening in septum primum (Text-Fig. 4, C) does not cease at the proper point, septum primum may be so extensively destroyed that it fails to occlude effectively a foramen ovale of normal size (Figs. 3 and 4). This is a radically different process from a developmental arrest. Instead of dealing with a growth process which has not gone to completion, we are dealing with a process of resorption that has gone too far.

Another condition which is a variant of the process just considered occurs not infrequently. The resorption of septum primum may take place in abnormal areas as well as to an abnormal degree. Instead of being limited to the quadrant in which the secondary opening in septum primum is ordinarily established, the resorption may occur in several places and progress to such an extent that the remains of septum primum can not possibly act as an efficient valve at the foramen ovale (Fig. 6). This abnormal resorption may start from the margins, as in the heart shown in Figure 6, or it may appear also in the form of multiple small openings reminiscent of the manner in which ostium II is first formed in septum I (Text-Fig. 4, C). The openings may be formed near the normal site of

ostium II or at various other parts of the valvula as shown in Figures 7, 8, 10, 12. In such cases we are dealing with the distortion of a resorptive process instead of with the "arrest" of a growth process. When it progresses and terminates normally, this process of resorption plays just as important a part in moulding an efficient valve as do the growth processes with which it is correlated.

In rare instances hearts are encountered that show no trace of a valve covering the foramen ovale (Fig. 16). While it is impossible to be certain that this represents a defect due to secondary resorption of a once present septum primum, circumstantial evidence points to that conclusion. When septum primum is primarily defective there remains a very characteristically shaped opening just above the atrioventricular valves. Usually the valves themselves are notched at the point where septum primum would have fused with the atrioventricular canal cushions. The absence of such a condition, in this heart, and the existence in other hearts of a whole series of conditions grading toward complete destruction of the free part of the valve (Figs. 1–14) point strongly toward secondary absorption of a once present septum primum as the correct interpretation.

The most common type of defect at the foramen ovale appears to be that in which there has been just a little too much resorption of septum primum at the normal site of ostium II (Fig. 3). This condition is surprisingly frequent in newborn infants. In a series of 100 consecutive cases studied with special reference to this condition its incidence was above 20 per cent. Apparently such slight failures of the valvula to overlap the foramen are rapidly compensated for in some manner because, except in newborn infants, they are not strikingly common. It may be that septum secundum grows somewhat after birth thereby reducing the extent of the foramen ovale and eliminating the small unguarded area. It is possible, also, that the marked fibrous development characteristic of the valvula from the 2nd to the 9th month after birth may account for the elimination of this defect in some cases. In 1 unusual case, secondary growth of the tissue around the limbus fossae ovalis was very marked and there had been, also, as far as one could judge by looking at the completed process, some secondary filling in of small multiple defects in the valvula (Figs. 20, 21, 22). How common such repair may be it is impossible to guess. The case

mentioned is the only one of the kind I have seen but it seemed unmistakable in its significance.

Probably the rarest of anomalies occurring at the foramen ovale is congenital atresia (Corvisart, 1818; Smith, 1846; Osler, 1880; and Lehman, 1927). Through the courtesy of Dr. Howard T. Karsner I had the opportunity of seeing the additional case illustrated in Figures 17, 18 and 19. Such cases throw an interesting side light on the functional significance of the foramen ovale during fetal life, for in every instance the left ventricle was developed to only about half its normal size. The muscular development of the ventricles being largely influenced by the volume of blood that they handle during their period of growth, one must infer that the half-normal development the left ventricle acquires in cases where the foramen ovale is prematurely closed depends on the blood returning to the left heart through the fetal lungs. The condition seen in these cases seems to corroborate the interpretation given above on the basis of orifice measurements, that approximately half the blood entering the left side of the heart in a term fetus comes by way of the lungs and half by way of the foramen ovale.

From the morphogenetic standpoint, congenital stenosis or atresia of the foramen ovale presents yet another different type of departure from the normal. It is not the result of inhibited growth, nor yet of exaggerated or distorted resorption. On the contrary, it is the continuation of a normal constructive process "beyond the point specified in the plans." Septum secundum fails to cease growing when its margins have reached the usual boundaries of the foramen ovale. Its growth continues abnormally until it has closed an opening without which the left side of the heart develops so defectively that it cannot long maintain the load imposed on it after birth.

That congenital defects at a given location may arise in such fundamentally different ways would seem to have significant implications. Cases have been here presented which show abnormal interatrial openings appearing as the result of: (1) underdevelopment of septum secundum; (2) resorption of septum primum starting in the normal location but going too far; (3) resorption of septum primum taking place in abnormal locations; and (4) overgrowth of septum secundum. Such radical differences in the

immediate mechanisms concerned should give us pause in considering any "blanket explanation" of congenital defects. Certainly the ultimate solution of the intricate problem of their causation will not be advanced by overemphasizing the developmental arrest concept when congenital defects may equally possibly be the result of a resorptive process which has gone astray, or a growth process which has failed to stop soon enough. Pending the acquisition of more satisfactory knowledge as to etiology, we would be on sounder ground if we were more restrained in our use of "developmental arrest" with its often false implications as to causation, and employed some such non-committal expression as developmental distortion or developmental defect.

Clinical Significance of Defects at the Foramen Ovale

It would carry me out of my province to undertake any extensive discussion of the clinical problems presented by individuals with congenital defects at the foramen ovale. There are, nevertheless, certain things that stand out from a study of the records of a large number of cases that it might not be out of place to mention.

The still rather widespread practice of attributing otherwise unaccounted for deaths of young infants to "an open foramen ovale" is utterly unsound. In the first place, anatomical closure of the foramen ovale does not ordinarily take place until toward the close of the 1st postnatal year. Secondly, from 20 to 25 per cent of all adults show incomplete fusion of the valvula to the septum without the slightest evidence that this condition is any functional handicap, provided the valvula adequately covers the foramen and there are no other concomitant circulatory disturbances. Finally, the way individuals with extensively unguarded interatrial openings frequently live into maturity and even old age clearly shows the absurdity of regarding a mere unclosed foramen ovale as the immediate cause of a fatal circulatory disturbance.

While an unclosed but competent valvula foraminis ovalis cannot be regarded as a causative factor in circulatory disturbances, it frequently is the result of disturbances elsewhere. If, during the period in which the valvula would normally fuse to the septum, there is any condition operative that reduces the left atrial intake from the lungs, transseptal blood flow from right to left will continue to take place postnatally as it did prenatally and the

valvula will thereby be prevented from fusing to the septum. The most striking cause of such a situation is congenital pulmonary stenosis. Almost without exception when there is a pulmonary stenosis of embryological origin, an unfused and slack valvula persists at the foramen ovale. This is the logical sequel of the failure to accomplish the balancing of direct atrial intakes which normally comes with the attainment of full functional activity by the pulmonary circuit.

While it may not invariably be the case, there is usually a recognizable morphological difference between the condition exhibited by a valvula which has been kept open by circulatory unbalance and one which, although subjected to no such disturbance, has failed to fuse with the septum. In the fortuitous failure of complete adhesion which is encountered in 20 to 25 per cent of all adults the valvula tends to lie tight against the septum and the areas of incomplete fusion may be entirely overlooked unless one meticulously explores all parts of the valve margins with a fine flexible probe. When transseptal blood flow has persisted, the valvula tends to retain a certain fullness like that characteristically present in the fetus. It is likely for the same reasons to lie less closely against the septum, thus readily revealing the presence of an interatrial communication. These differences appear most clearly when a fresh heart is examined under water, with the aid of a current from a syringe.

There is a rather neglected corollary to the proposition that a foramen ovale with a freely opening valve should be regarded as part of the picture one would expect to find with congenital pulmonary stenosis. Cases of pulmonary stenosis not infrequently come to autopsy in which conditions at the pulmonary outlet alone do not give satisfactory evidence as to whether the stenosis was congenital or acquired. In such cases conditions at the foramen ovale may furnish valuable collateral evidence. In the absence of some other circulatory by-pass of similar functional significance, a completely closed foramen ovale in a case of pulmonary stenosis is strong evidence that the stenosis developed after the fetal-neonatal period.

Thus it should be emphasized that, when the foramen ovale possesses a competent valve, the question of whether or not this potential by-pass is closed should be considered in the light of the

factors involved in establishing and maintaining a balanced atrial intake. Phrased in another way, an open foramen ovale of this type is not to be thought of as a cause of circulatory disturbances but as a result of them. Equally obviously, the question of the condition of the pulmonary circuit should be the first consideration from the standpoint of diagnosis. The fundamental importance of maintaining a right to left transatrial blood flow during fetal life is correlated with the low volume of the pulmonary circuit while the lungs are not functioning in respiration. The cessation of transatrial blood flow and the balancing of direct atrial intakes, which occurs following birth, is dependent on the attainment of full functional level in the pulmonary circuit. And finally there is no single factor as likely as some disturbance of the pulmonary circulation to upset this balance during postnatal life and thus reopen a foramen ovale which has happened to remain unsealed.

A heart with a foramen ovale which is inadequately guarded by a malformed valvula (Figs. 14 and 15), or one which is completely unguarded (Fig. 16) presents quite different possibilities from cases of the type just discussed. In hearts with a competent but unfused valvula, transseptal flow takes place readily from right to left but is inhibited from left to right. When there is an unguarded foramen ovale, transseptal flow can take place just as readily from left to right as from right to left. Of course if there is an associated pulmonary stenosis causing a compensatory flow from right to left, the mechanism of the circulation will be quite similar to that just discussed for a heart with a competent but unfused valvula. If, on the other hand, the pulmonary circuit is normal a radically different picture is presented. The clinical manifestations of such cases have recently been presented very ably and in considerable detail by Roesler (1934). When, as Roesler has done, all cases of interatrial defect in which there is an associated pulmonary stenosis are ruled out, a very characteristic picture remains. Its outstanding features, summarized from Roesler's findings, are essentially as follows: The heart tends to be enlarged, often becoming of enormous size and causing a marked precordial bulge. The enlargement involves primarily the right side of the heart and especially the right ventricle which is usually both extensively dilated and hypertrophied. The left ventricle remains strikingly uninvolved by the enlargement (Abbott's Case

1, 1915, with hypertrophy involving the left as well as the right ventricle is exceptional). The pulmonary artery is consistently larger than the aorta, the average ratio being 3:2. The aorta tends to be below normal size and thin walled. In upwards of three-fourths of the long-standing cases valvular lesions were found which affected predominantly the mitral orifice (see also McGinn and White, 1933). In contrast with certain other types of congenital defects, subacute bacterial endocarditis is strikingly absent, and chronic pericardial disease, crossed embolism, and pulmonary tuberculosis are noticeably rare concomitants.

The critical factor in this picture again appears to involve relative atrial intakes and pressures. In normal adults the pressure in the left atrium is believed to be slightly greater than that in the right. Under such conditions an unguarded interatrial opening would permit the backing of blood from the left atrium into the right, thus causing the right side of the heart to handle an increased amount of blood. This situation acting over a long period of time would account for the dilatation and hypertrophy of the right ventricle and the large pulmonary arteries which are so characteristic in these hearts. The same transatrial flow which overloads the right heart, by reducing the blood entering the left ventricle, would account for the fact that it is consistently uninvolved by dilatation or hypertrophy, and also for the fact that the aorta tends to be relatively small.

Detailed physiological evidence for this interpretation is admittedly scanty but the circumstantial evidence is convincingly consistent. For a fuller discussion from a clinical standpoint reference should be made to Roesler's excellent paper. There are, however, two points of special interest which it might be pertinent to mention here. One is the striking absence of crossed emboli in cases of frankly unguarded interatrial openings where the flow is presumably taking place from left to right, in contrast with the comparative frequency of crossed emboli in association with pulmonary stenosis where the interatrial communication may be much smaller but where the leakage is from right to left. The other point concerns cyanosis. Characteristically, as long as the individual is subject to no complicating factors, cyanosis will be absent as one would expect on the basis of the above interpretation of a left to right shunt. A sudden terminal cyanosis ("cyanose tardive,"

Bard and Curtillet, 1889) is, however, very likely to occur. What apparently happens in such cases is a reversal of the direction of the shunt due either to intercurrent pulmonary difficulty, or to breakdown of the long overloaded right ventricle.

Many individuals, even with large interatrial defects, live to advanced years with surprisingly little handicap. Perhaps the most interesting are cases in which individuals have performed hard work for many years or otherwise lived an active life. Caton (1878) wrote of a man, powerfully built, who had been a seaman for 20 years and finally died of an acute respiratory infection at the age of 40. His interatrial defect was 3 inches in diameter. The heart here illustrated in Figure 14 was from a charwoman who lived to the age of 52, and that illustrated in Figure 16 was from a day laborer who lived to the age of 44. Gibier (1880) records the case of a man who showed no cardiac symptoms during life, withstood anesthesia for a cancer operation and lived to the age of 70. Firket (1880) reported on a woman who had 11 children, and lived to an age of 74 years. Tarnower and Woodruff (1936) have published the clinical findings and detailed autopsy report on a woman who lived to be 77 years of age although she had a patent foramen ovale measuring 4 cm. in diameter. Why this tolerance of the defect is so striking in some cases and why other individuals with similar defects are incapacitated and go on to a relatively early death from cardiac failure is difficult to explain. One significant fact brought out by Roesler, is that in three-fourths of the 62 cases of unguarded interatrial defects which he reviewed chronic valvular lesions of some degree were found. This is considerably above the average incidence of such lesions in all types of congenital defects, which is placed by Abbott (1932) at 17.6 per cent. The mitral orifice seems to be the one most frequently affected in individuals with interatrial defects. While there is no clue as to why this is the case the aggravating results of its occurrence are self-evident. Either mitral insufficiency or stenosis will tend to exaggerate the flow from left atrium to right which is the critical factor in producing the characteristic cardiac changes seen with an interatrial defect, whether it be at an unguarded foramen ovale or at the site of interatrial foramen primum. Apparently when this back flow becomes extreme there is a breakdown of the compensation previously maintained and the individual begins to show

marked cardiac symptoms which are likely to increase rather rapidly in severity.

As far as it is possible to generalize, the situation would appear to be that even a large interatrial defect is by itself not necessarily incompatible with a long and active life. It does, however, produce striking and characteristic changes in the proportions of the heart. Moreover, such hearts appear to be more than normally vulnerable. Individuals who have carried the defect for years without particular handicap may suddenly show signs of cardiac failure. What part is played in such cases by the frequently concomitant valvular disease, why valvular disease has such a high incidence in these hearts, and why it shows a predilection for the mitral orifice are all matters that need further study.

Summary

This paper aims to present a survey of the various types of developmental defects encountered at the foramen ovale, and to make a re-evaluation of their significance. As a foundation for this study the formation of the interatrial septal complex in the embryonic heart is reviewed. In this review special attention is given to the functional significance of the three different interatrial communications which appear during intrauterine life.

The mechanism of the closure of the foramen ovale following birth, and the time at which its closure may be expected to occur, are presented in the light of recent work which points to the conclusion that the postnatal changes in circulation are far less abrupt and immediate than traditionally postulated.

The various types of developmental defects which may occur at the foramen ovale are illustrated and the embryological processes which have been distorted in the production of each type of defect are discussed. It is pointed out that the "developmental arrest" concept is inadequate as an interpretation, in view of the several fundamentally different ways in which the developmental defects may arise.

Finally, brief comment is made on certain things of clinical interest that emerge from the review of a large number of cases. Reasons are given for regarding as unsound the still widespread practice of attributing otherwise unaccounted for deaths of neonatal infants to "an open foramen ovale."

Emphasis is placed on the necessity of discriminating more critically between an open foramen ovale with a competent but unfused valve, and a frankly unguarded foramen ovale with an incompetent valve. With an unfused but competent valve transseptal leakage, if it occurs at all, is limited to one direction — right to left. The common cause of such leakage is some disturbance of the pulmonary circuit which results in relative lowering of left atrial intake and pressure. What occurs at the foramen ovale in such cases should be regarded as a result of disturbances elsewhere and not as a cause.

In sharp contrast with such cases are those in which the foramen ovale is inadequately guarded by an incompetent valve. In these cases, provided the pulmonary circuit is normal, transseptal flow appears to take place quite consistently from left to right. This overloads the right heart at the expense of the left and causes characteristic changes from the normal cardiac proportions. There tends to be a marked dilatation and hypertrophy resulting in a great increase in heart weight. The right side of the heart, especially the right ventricle, is most conspicuously involved, whereas the left ventricle remains strikingly uninvolved. Consonant with the relative ventricular development, the pulmonary artery is markedly larger than the aorta, which tends to be below normal size and thin walled. In these cases with an unguarded foramen ovale the characteristic and clinically recognizable changes in cardiac structure are clearly the result of the defect. Even in these cases the prognosis should not be unduly pessimistic as many individuals support such defects surprisingly well and live to an advanced age.

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DESCRIPTION OF PLATES

PLATE 30

FIG. 1. Usual appearance of the valvula foraminis ovalis as seen from the left in the heart of a newborn infant. Note the fullness of the valvula which is represented in the bulged out position it assumes when subjected to excess fluid pressure from the right atrium through the foramen ovale. The size and position of the foramen ovale are indicated by the broken line.

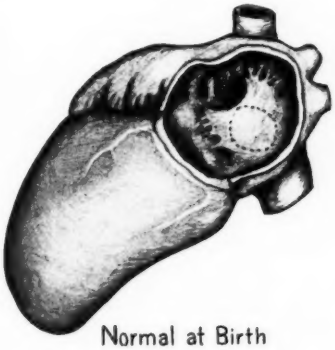
FIG. 2. Resorption of septum primum in an abnormal area dorsal to the usual site of ostium secundum. In this instance the abnormality is of no functional significance since it does not unguard the foramen ovale.

FIG. 3. Slight incompetence of valvula foraminis ovalis due to excess resorption of septum primum at the normal site of ostium secundum. This is a common condition, occurring in some 20 per cent of newborn infants. It is apparently "corrected" in most cases by postnatal changes either in septum primum or septum secundum and probably has no functional significance.

FIG. 4. Incompetence of valvula due to excess resorption at the normal site of ostium secundum combined with resorption at an abnormal site. This defect is definitely more extensive than the apparently correctable type shown in Fig. 3, and undoubtedly would persist throughout life.

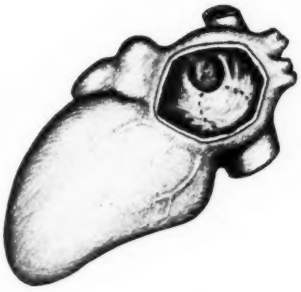
FIG. 5. The valvula is abnormally resorbed in two small areas but these defects are so slight that they would be of no significance were they not combined with an abnormally large foramen ovale. The large foramen ovale due to defective development of septum secundum is the condition of primary importance in this case.

FIG. 6. Extensive defects of the valvula due to over-resorption in the normal and in several abnormal locations.



1 Normal at Birth

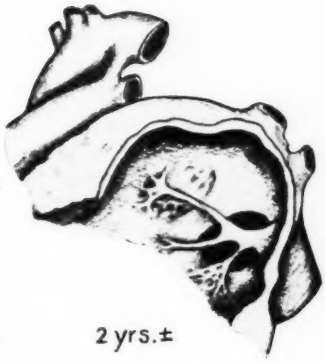
2 12 days ♀



3 7 wks. ♂

4 Stillborn ♂

5 16 hrs. ♀



6 2 yrs.±

PLATE 31

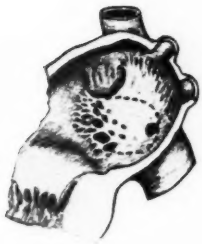
FIG. 7. Multiple small perforations of valvula. The formation of ostium secundum normally starts with the appearance of small openings which later coalesce. Here such openings have appeared in a definitely abnormal location.

FIG. 8. Case similar to that shown in Fig. 7, except that the openings are larger and more widely distributed.

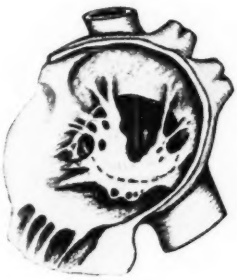
FIGS. 9, 10 and 11. Various combinations of marginal over resorption of the types shown in Figs. 1–6 with small multiple perforations similar to those shown in Figs. 7 and 8.

FIG. 12. Extreme resorption of valvula combined with abnormally large foramen ovale due to defective development of septum secundum. There is also in this heart unbalanced development of the ventricles, the left ventricle being very small, correlated probably with a defective pulmonary circuit as indicated by the marked stenosis of the pulmonary veins.

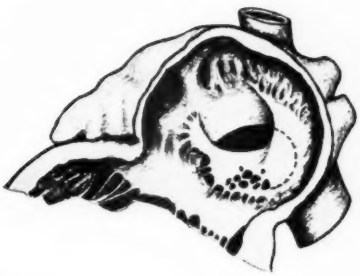
7 3 mos. ♂



8 Stillborn ♀

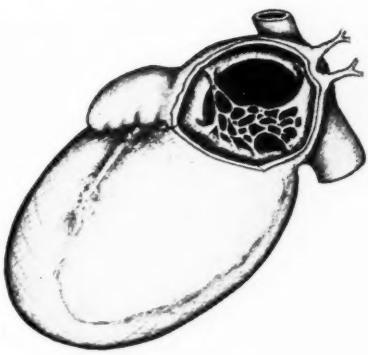


9 4 mos.±



10 5 mos.±

11 6 wks. ♀

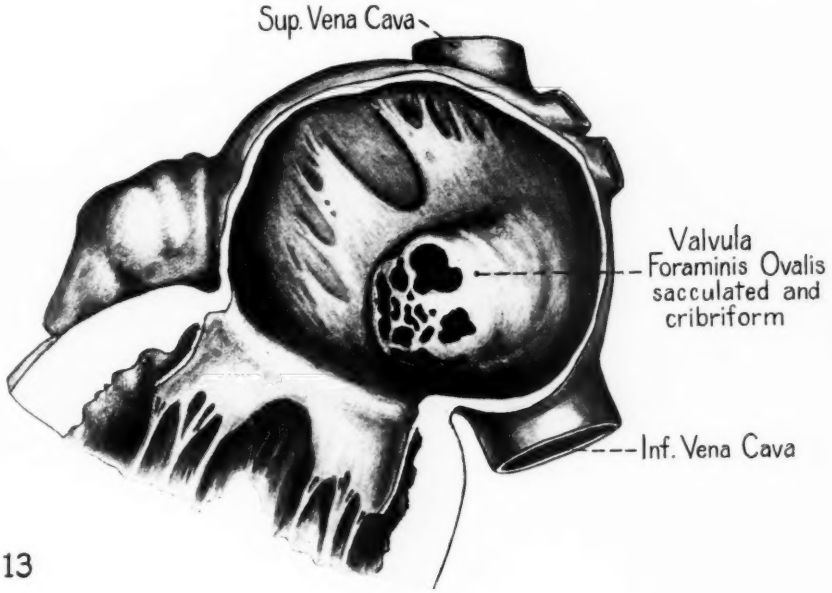


12 Stillborn ♀

PLATE 32

FIG. 13. Valvula foraminis ovalis markedly sacculated toward left atrium and showing multiple perforations of considerable size. No clinical history. Dissecting room specimen "from an old man" sent in by Dr. John Donaldson, University of Pittsburgh.

FIG. 14. Drawn from specimen No. 3027, Pathologisch-Anatomisches Institut, Vienna. The heart was from a charwoman who died suddenly of pulmonary thrombosis at the age of 52 years. Rokitansky (1875, p. 52) gives a brief unillustrated record of the case. The heart was "very large, 90 mm. long and 115 mm. broad" with rounded apex. The similarity of the morphological picture presented by this adult heart and the infant heart shown in Fig. 11 is interesting.



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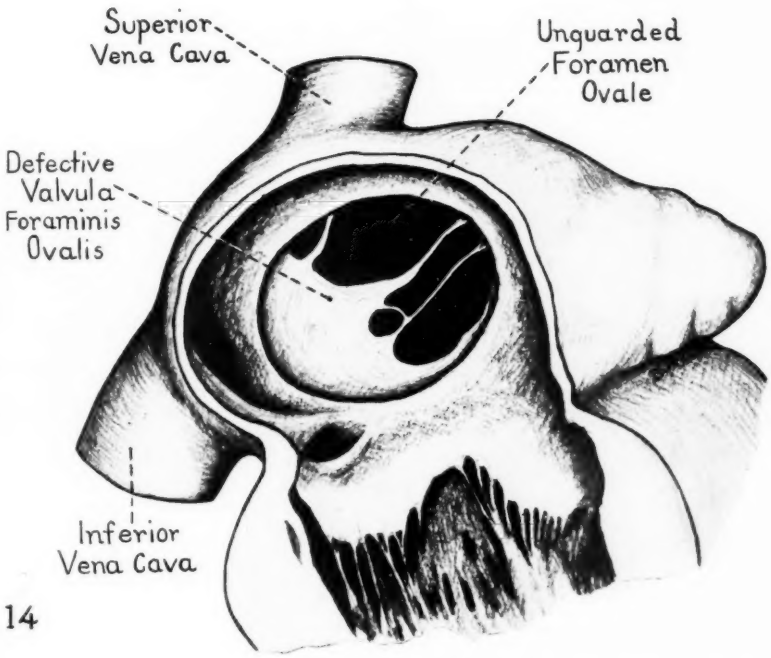

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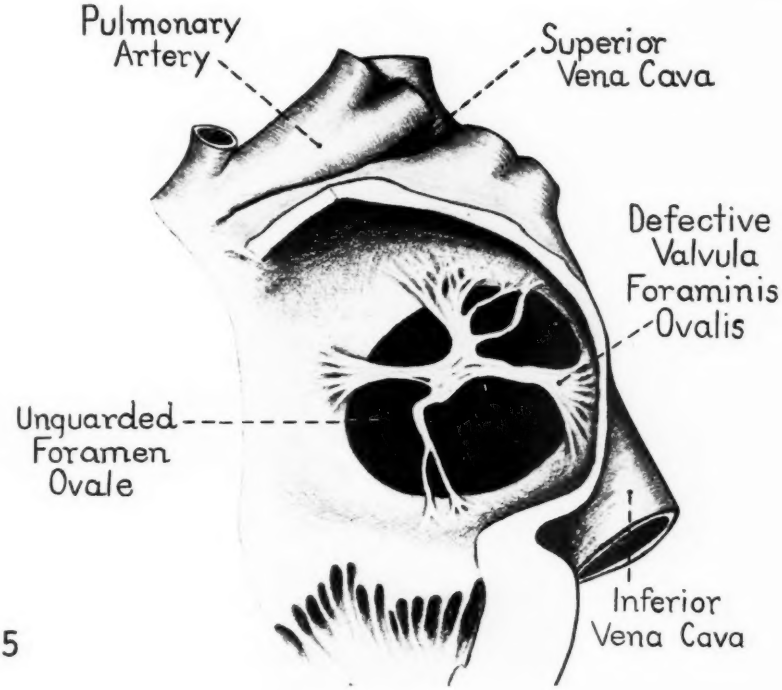
Plate 33

Fig. 15. Drawn from specimen No. 2410, Pathologisch-Anatomisches Institut, Vienna. Case briefly described by Rokitansky (1875, p. 47). Day laborer 21 years old, admitted to the hospital with "the itch" (Krätze). Died following an unexpected attack of dyspnoea. Heart very large, "100 mm. lang und ebenso breit"; right ventricle and conus "erweitert." Heart weight not recorded.

Fig. 16. Drawn from specimen No. 2225, Pathologisch-Anatomisches Institut, Vienna. Case mentioned briefly by Rokitansky (1875, p. 45). Male, day laborer, 44 years old. Had purulent bronchitis and gangrene of oral mucous membrane. No cyanosis noted. Immediate cause of death appeared to be primarily pulmonary, although the clinical information given is too meager to be certain. The heart was greatly enlarged and showed a fibrinous pericarditis. No trace of a valvula foraminis ovalis could be seen and the unguarded foramen ovale was of enormous size measuring 40 by 47 mm. in the fixed specimen.



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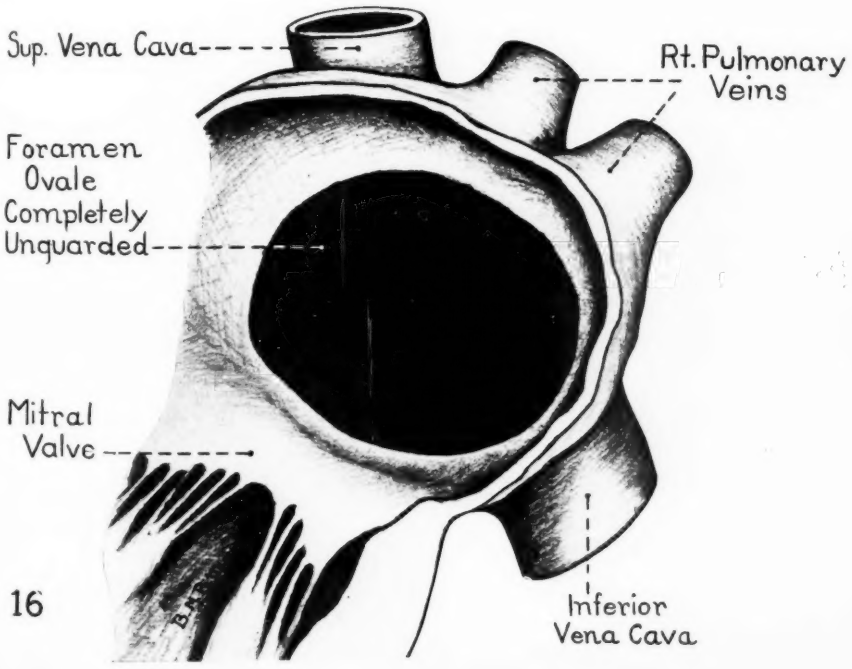

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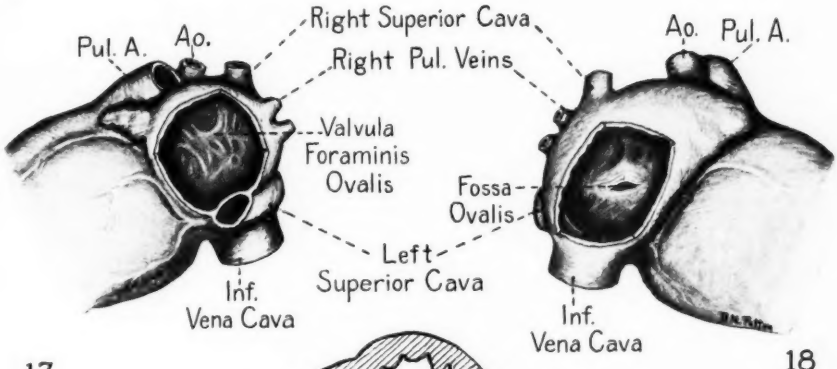
PLATE 34

FIGS. 17, 18 and 19. Heart of a 1 month old infant showing the conditions encountered in premature closure of the foramen ovale. (Babies and Childrens Hospital, Autopsy No. A–374, made available through the courtesy of Dr. Howard T. Karsner, Western Reserve University Medical School.) Although there is no way of being certain whether the closure as seen at autopsy had been fully established *in utero*, there seems no doubt that a marked ante natal stenosis, if not an atresia, must have existed. This is indicated: (1) by the slit-like fossa ovalis which is but a small fraction of the oval opening left when septum secundum normally ceases further growth; (2) by the complete adhesion of the valvula which does not ordinarily occur until several months after the cessation of transseptal flow; and (3) by the deficiently developed left ventricle which seems clearly attributable to lessened left atrial intake due to a foramen ovale closed, or greatly narrowed, during the growth of the fetal heart.

FIGS. 20, 21 and 22. Possible case of postnatal repair of congenital defect at the foramen ovale. The appearance of the narrowed fossa ovalis is superficially somewhat similar to the case of premature closure illustrated above, but there are two associated conditions which indicate that in this case the narrowing occurred postnatally. First is the normal development of the ventricles. If the fossa ovalis had been of its present abnormally small size during intrauterine life the left ventricle would have been undersized. Second is the faint depression which sketches the contours of a fossa ovalis of the normal size. This seems to suggest that the fossa was, at the time of birth, of the size outlined by this depression, and that the differently disposed tissue now narrowing it was formed later. There is yet another interesting condition pointing in the same direction. In the valvula foraminis ovalis one sees an arrangement of robust strands which suggest that it might once have appeared not unlike the defective valve in Fig. 9. Between these heavy strands there is a more delicate tissue which might conceivably have been secondarily formed. Of course this entire interpretation must be regarded as tentative, but postnatal repair of a congenital defect, if it does occur, is of so much interest that it seemed worth while presenting this unique case in the hope of stimulating further observations bearing on such a possibility.



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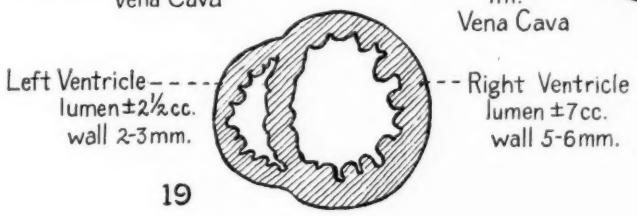

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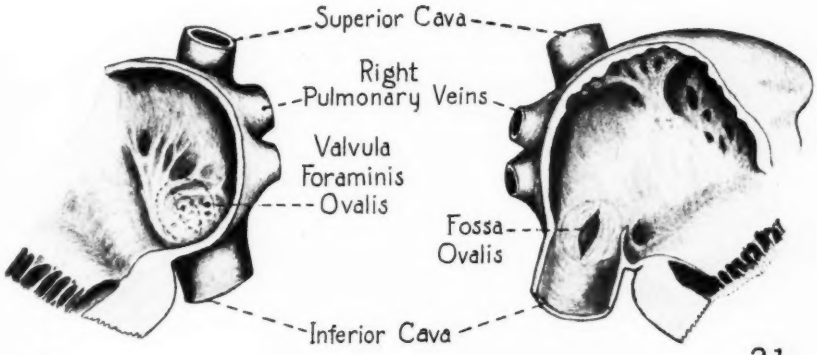
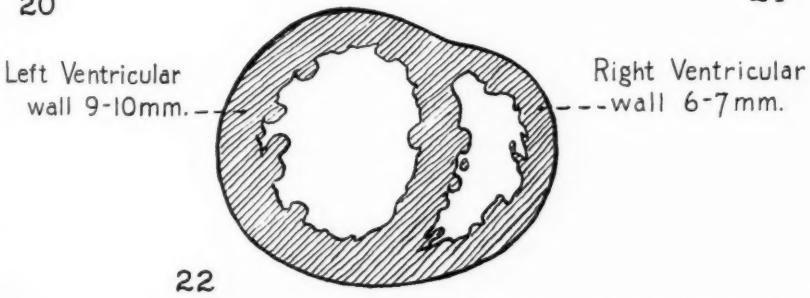


20 21



22

THE EFFECT OF SYPHILIS ON LOCAL TUBERCULOUS LESIONS IN RABBITS *

J. D. Aronson, M.D., and D. R. Meranze, M.D.

(From the Henry Phipps Institute, University of Pennsylvania, and the Mount Sinai Hospital, Philadelphia, Pa.)

The influence of syphilis on the course of other diseases, and other diseases on the course of syphilis, is not definitely known. Certainly the view held by John Hunter [1] that "no two actions can take place in the same constitution or in the same part at once and at the same time" is not tenable in the light of our present knowledge. Pearce [2] observed that the simultaneous inoculation of vaccine virus and syphilitic material into two different sites resulted in a more severe form of syphilis in rabbits, whereas the injection of the syphilitic material into rabbits previously immunized with vaccine virus gave rise to an infection with a less severe course. Pearce and Brown [3] found that with one exception a transplantable malignant tumor of rabbits failed to grow in rabbits infected with syphilis. Chesney and Kemp [4] noted that inflammation induced by trauma or by coal tar favored the multiplication of the *Treponema pallidum* and the extension of the syphilitic process.

As to the part played by syphilis in tuberculosis, there is a wide difference of opinion. Sergent [5] believed that syphilis predisposes to tuberculosis and creates a site of predilection for the tuberculous process. That active syphilis influences unfavorably the course of tuberculosis has been maintained by Norris and Landis,[6] Fishberg,[7] Habliston and McLane,[8] Chadwick,[9] and others. On the other hand, Petresco [10] was of the opinion that there is a definite antagonism between the two. Weiss [11] could not establish any significant clinical relation between syphilis and tuberculosis. He concluded that the influence of syphilis on tuberculosis varies with the degree of the constitutional resistance of the individual.

Experimental

In order to determine whether syphilis influences the course of tuberculous lesions, a study of the gross and microscopic character

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of tuberculous lesions of the skin of syphilitic and of non-syphilitic rabbits was undertaken. This method of approach was chosen because it permitted observation of lesions in the same animals at varying intervals over a considerable period of time, thus minimizing the element of individual variation in resistance.

Method

Into the left testicle of each of 12 albino New Zealand rabbits, weighing from 2000 to 2500 gm., an emulsion of the testicle of a rabbit that had previously been infected with the Nichols strain of *Tr. pallidum* * was injected. On dark-field examination this emulsion showed 4–5 spirochetes per high power microscopic field. Three to 4 weeks following inoculation the characteristic enlargement and swelling of the testes were observed, followed by prompt subsidence of the swelling and the appearance of indurated nodules. In all of the inoculated rabbits metastatic lesions were found in the opposite testicle. Nine weeks following the infection with *Tr. pallidum*, an injection of 0.1 mg. of a virulent strain of bovine type tubercle bacillus in 0.1 cc. of physiological salt solution was made into the skin over the abdomen at each of six widely separated points. At the same time 12 non-syphilitic rabbits of approximately the same weight and breed were injected in precisely the same manner with a similar amount of the bacillary suspension. All of the rabbits were maintained on a diet of prepared pellets, hay and water, with fresh greens twice a week. All animals continued to gain in weight until the 12th week, when a marked loss of weight was noted.

The resulting tuberculous lesions were studied macroscopically, and were then biopsied under ether anesthesia for histological study according to the following scheme: In a series of 3 syphilitic and 3 non-syphilitic rabbits a lesion was removed from each rabbit 1, 3, 5, 12, 24 and 48 hours after the injection of the tubercle bacilli; in a second series of 3 syphilitic and 3 non-syphilitic rabbits lesions were removed after 3, 4, 5, 6, 7 and 14 days; in a third series, specimens were taken after 3, 4, 5, 6, 7 and 8 weeks; and in a fourth series, after 9, 10, 11, 12, 13 and 14 weeks. A section of uninoculated skin was removed from each rabbit as a control.

* This strain was kindly furnished us by Dr. M. Severac of the Dermatological Research Laboratory, Philadelphia.

The sections removed at biopsy were embedded in paraffin, sectioned and stained with various stains, including hematoxylin and eosin, Ziehl-Neelsen stain for acid-fast bacilli and Mallory's aniline blue collagen stain.

Macroscopic Characteristics of Lesions

The local tuberculous lesions were found to vary in size, not only among the different rabbits but also at the different sites of injection in the same rabbit.

Tubercle formation occurred earlier and was, on the average, throughout the course of the experiment, from 2 to 5 mm. larger in diameter and more elevated in the syphilitic rabbits. In an occasional syphilitic rabbit, however, the lesion was no greater than that noted among the controls. Ulceration, which occurred among the non-syphilitic rabbits within the 1st week, did not occur until the 2nd week among those with syphilis. These ulcers, which were 2 to 8 mm. greater in diameter among the syphilitic animals, extended more rapidly and showed less tendency to heal than similar ulcers in the non-syphilitic. In both groups the ulcers tended to be sharply defined and crater-like, with undermined edges.

The variation in the time of enlargement, in size and in the occurrence and extent of caseation in the regional axillary and inguinal lymph nodes, was so great among individual rabbits as not to permit drawing any conclusions.

Pathological Histology

Sections of the uninoculated skin removed from the syphilitic and from the non-syphilitic rabbits showed no conspicuous histological differences.

Sections of the skin removed from both syphilitic and non-syphilitic rabbits 1 hour after the injection of 0.1 mg. of tubercle bacilli showed a moderate degree of swelling in the derma, some separation of the collagen fibers, and dilatation of blood vessels. Slight leukocytic infiltration, most marked in the papillary layer of the derma, and somewhat more intense in the syphilitic than in the non-syphilitic rabbits, was present. Polymorphonuclear cell phagocytosis of the tubercle bacilli, which at this period appeared singly and in small clumps, was noted in both groups of animals.

Sections of skin removed from the non-syphilitic rabbits 3 hours, 5 hours and 12 hours after inoculation showed in the widened and edematous tissue spaces a diffuse but moderate degree of infiltration with polymorphonuclear cells, as well as small aggregations of these cells about clumps of tubercle bacilli. The blood vessels were dilated, and a slight degree of diapedesis was observed. Phagocytosis of tubercle bacilli by polymorphonuclear cells was evident and numerous extracellular clumps of these microorganisms were seen.

In the sections of skin removed from the syphilitic rabbits 3 hours, 5 hours and 12 hours following the injection of tubercle bacilli into the skin, the blood vessels and lymphatics were markedly dilated and the degree of edema was greater than among the non-syphilitic rabbits. Numerous leukocytes were observed along the endothelial lining of the vessel wall, while dense aggregations of polymorphonuclear cells and amphophiles occurred about the vessels (Fig. 1). The lymphatic vessels were dilated and contained small numbers of lymphocytes in contradistinction to the lymphatic vessels of the non-syphilitic rabbits, which were inconspicuous. Phagocytosis of tubercle bacilli by polymorphonuclear cells was not conspicuously different from that observed in the non-syphilitic animals, except that in sections removed from the syphilitic rabbits 12 hours after infection, was observed a marked increase of mononuclear cells, many of which were phagocytic.

In sections of skin removed from the non-syphilitic rabbits at daily intervals during the 1st week following infection an increasing number of polymorphonuclear cells was observed, and amphophiles and some mononuclear cells of the macrophage type were seen scattered diffusely throughout the derma and subcutaneous tissue, and occurring occasionally as small aggregations about blood vessels, as well as about clumps of tubercle bacilli. Three to 4 days after infection there was noted a small abscess which rapidly extended and which several days later ulcerated through the epidermis. The abscess consisted of dead and degenerated cells and collagen fibers (Fig. 2). Numerous tubercle bacilli, many extracellular and some intracellular, occurring in clumps, as well as dispersed, were noted within the abscess and to a lesser degree in other parts of the lesion. The abscess was surrounded by a moderate number of newly formed capillaries and a moderate dif-

fuse infiltration of mononuclear cells, amphophiles and fibroblasts.

In the lesions removed from the syphilitic rabbits during the 1st week following infection with tubercle bacilli, the histological changes were significantly different from those observed in the non-syphilitic rabbits. In these animals there were observed more or less widely separated, sharply defined aggregations of mononuclear cells massed about vessels and in some instances about newly formed capillaries (Fig. 3). These mononuclear cells had large, round, pale staining vesicular nuclei which, in some cells, showed indentations. An occasional polymorphonuclear cell and amphophile were observed in these aggregations. Between the collagen fibers were noted an increased number of fibroblasts as well as some infiltration with mononuclear cells. The blood vessels were dilated and numerous polymorphonuclear cells and amphophiles were observed along the endothelial lining.

The above described cell aggregations were first observed 48 hours after the injection of tubercle bacilli and became increasingly denser during the 1st week. In the syphilitic rabbits abscess formation was delayed, occurring 1 to 2 weeks after infection, and was less extensive than that noted among the non-syphilitic animals.

These lesions, because of the focal aggregations of large mononuclear cells about capillaries and the formation of fibroblasts and capillaries, resemble the lesions of primary and secondary syphilis. Figure 4 is a microphotograph of a primary lesion resulting from the injection 6 weeks previously of a suspension of *Tr. pallidum* into the skin of a normal rabbit.

In the lesions removed from the non-syphilitic rabbits 2 weeks after infection with tubercle bacilli there was an abscess that had ulcerated through the epidermis and was sharply defined by a zone of necrosis beneath which granulation tissue rich in capillaries and fibroblasts was present. A small number of epithelioid cells and some plasma cells were scattered throughout the granulation tissue. No acid-fast bacilli were found after prolonged search. In the lesions removed a week later the abscess and ulcer had extended and widespread edema separated the dead and degenerating polymorphonuclear cells from one another. Beneath the area of ulceration there was a diffuse infiltration with epithelioid cells, many of them with a large amount of foamy cytoplasm.

In the lesions removed from the syphilitic rabbits 2 weeks after infection, ulceration was more extensive than among the non-syphilitic rabbits and the slough was separated from the underlying tissue by a zone of deeply staining polymorphonuclear cells. Beneath this zone there was an extensive infiltration of mononuclear cells resembling those previously described in the syphilitic rabbits. These cells, many of which showed mitotic figures, occurred in aggregations among the collagen fibers. Similar aggregations occurred about the dilated vessels and about some of the capillaries. An occasional small aggregation of epithelioid cells was seen throughout the lesion, but acid-fast bacilli were not demonstrable. In the lesions removed a week later the slough was definitely more extensive and granulation tissue was more conspicuous. Epithelioid cells occurred in moderate numbers, not diffusely as in the non-syphilitic rabbits, but in aggregations. A number of small areas of caseation were observed scattered throughout the deeper layers of the derma.

Four weeks after infection increasing ulceration was present in the lesions removed from the non-syphilitic rabbits. Beneath the newly regenerating epithelium, which dipped downward into the derma and into the subcutaneous tissue, there was a diffuse infiltration of epithelioid cells of varying size (Fig. 5), many of which contained protoplasm of foamy appearance. Occasional small aggregations of lymphocytes and an occasional giant cell, as well as small collections of plasma cells, were scattered throughout the lesion. The walls of the blood vessels were thickened. No tubercle bacilli were demonstrable.

In the lesions removed from the syphilitic rabbits 4 weeks after infection the ulceration was more extensive than among the non-syphilitic rabbits and no regeneration of the surface epithelium was noted. A well defined layer of polymorphonuclear cells was present beneath the ulcerated area and adjoining this layer was an area of granulation tissue rich in fibroblasts and mononuclear cells (Fig. 6). In the deeper layers of the derma there was a marked increase of dense connective tissue, while the blood vessels were definitely thickened, but to no greater degree than among the non-syphilitic animals. Epithelioid cells as well as mononuclear cells were distributed, not diffusely, but rather as dense aggregations, frequently about blood vessels and separated from one another by

dense bands of collagen fibers. Several small areas of caseation were noted, as well as small aggregations of plasma cells and a small number of giant cells. No tubercle bacilli were demonstrable.

In the lesions removed from the non-syphilitic rabbits 5 and 6 weeks following infection with tubercle bacilli, the surface ulceration, while extensive, was less so than in the previous lesions. Regeneration and invagination of the surface epithelium were again noted. A single, sharply defined large tubercle consisting essentially of epithelioid cells, and occasional collections of small lymphocytes were seen beneath the ulcer. In the deeper layers of the derma as well as in the subcutaneous tissue there were areas of granulation tissue and a marked increase of collagen fibers. Several small caseous areas were seen, as well as occasional areas of softening infiltrated in some instances with polymorphonuclear cells, many of which were degenerated. A number of giant cells were found while plasma cells were numerous throughout. Tubercle bacilli were not demonstrable.

In the lesions removed from the syphilitic rabbits at this time the surface ulceration had progressed, while there was little or no regeneration of the surface epithelium. Extensive granulation tissue consisting of newly formed capillaries and fibroblasts was observed throughout the derma and to a lesser extent in the subcutaneous tissue. Aggregations of large mononuclear cells and plasma cells were present about some of the newly formed capillaries. Areas of caseation more extensive than among the non-syphilitic rabbits were noted, and in some of the animals there were small areas of softening infiltrated with degenerating polymorphonuclear cells. Epithelioid cells were few, and usually found at the margin of the granulation tissue. These cells, smaller than those observed in the non-syphilitic rabbits, frequently occurred in aggregations about blood vessels. Plasma cells and giant cells were numerous, the former occurring in small dense aggregations. No tubercle bacilli were found.

The lesions removed at weekly intervals from the non-syphilitic rabbits 7 to 14 weeks after the injection of tubercle bacilli into the skin presented much the same histological characteristics as those observed 5 to 6 weeks after infection with tubercle bacilli. Surface ulceration tended to become smaller with increasing age of the lesion and in nearly every instance was covered by regenerated

epithelium which occasionally grew downward. A single, large, sharply defined tubercle was noted, consisting essentially of epithelioid cells with some plasma cells and surrounded by connective tissue which gradually increased in density (Fig. 7). The epithelioid cells contained an eccentrically placed nucleus, poor in chromatin, and with a pale staining foamy cytoplasm. A sharply defined, rounded, paler staining zone was frequently noted in the cytoplasm immediately beneath the nucleus. In the deeper and papillary layers of the derma and in the subcutaneous tissue areas of granulation tissue rich in plasma cells were present. The vessel walls continued to increase in thickness. Small aggregations of plasma cells occurred throughout the tubercle and to a greater degree between the connective tissue fibers. Lymphocytes were relatively inconspicuous until 9 weeks after infection, when small aggregations were noted, most frequently at the margin of the tubercle.

Small areas of caseation, frequently infiltrated with dead or degenerating polymorphonuclear cells, were observed in the deeper part of the tubercle, while more extensive areas of caseation were noted in the papillary layer of the derma. Extensive softening of the caseous areas was noted about 7 weeks after infection and continued to extend throughout the period of observation.

Giant cells were observed with increasing frequency during this time. These were found most frequently in the papillary layer of the derma. Tubercle bacilli were again noted within mononuclear cells from the 7th to the 12th week following infection, while no tubercle bacilli were demonstrable 13 and 14 weeks after infection.

In the lesions removed from the syphilitic rabbits at weekly intervals from the 7th to the 14th week inclusive following the injection of bovine type tubercle bacilli into the skin, the surface ulceration was more extensive than among the non-syphilitic rabbits and no regeneration of the epidermis had taken place. The surface ulceration was separated from the underlying tissue by a line of demarcation consisting of polymorphonuclear cells, many of which had degenerated. Beneath the surface ulceration and throughout the derma there were aggregations of mononuclear cells with large, rounded or elliptical nuclei, rich in chromatin and surrounded by pale staining protoplasm. There were also small numbers of young epithelioid cells and plasma cells. These aggre-

gations were surrounded by tissue rich in young fibroblasts as well as by dense connective tissue.

Beginning 8 weeks after infection with the tubercle bacilli there was, in the syphilitic animals, an increasing number of mature epithelioid cells within the previously described aggregations of mononuclear cells. These epithelioid cells did not differ morphologically from those previously noted in the non-syphilitic rabbits. On the other hand, there was a marked difference in the distribution and arrangement of these cells. In contradistinction to the single large epithelioid tubercle noted in the non-syphilitic rabbits, among the syphilitic animals there were numerous epithelioid tubercles separated from one another by more or less dense bands of connective tissue. There was a marked increase of dense connective tissue in the deeper layers of the derma as well as in the subcutaneous tissue (Fig. 8). The walls of both small and large vessels were thickened but to no greater degree than among the non-syphilitic rabbits. Small areas of caseation, in some instances infiltrated with degenerated leukocytes, were noted. These areas of caseation, occurring within the nests or aggregations of epithelioid cells, became more numerous and more extensive, resulting in the confluence of these areas, with the formation of large, caseous masses, much greater in extent than among the non-syphilitic rabbits. Softening was not observed until 13 weeks after infection.

In these rabbits, during the period from 8–13 weeks after the infection, small collections of plasma cells, in some instances about blood vessels and between connective tissue fibers, as well as small collections of lymphocytes, were present. The number, arrangement and morphology of the giant cells were not conspicuously different in the two groups of animals during this period. Tubercle bacilli were not observed in the sections removed at weekly intervals from the 7th to the 10th week inclusive. An occasional cell containing some tubercle bacilli was seen in the lesions removed 11 weeks and later following infection with the tubercle bacilli.

Summary, Discussion and Conclusions

These experiments indicate that syphilitic rabbits react in a different manner to the injection of living virulent tubercle bacilli than do similarly infected non-syphilitic rabbits. In the syphilitic

rabbits the local inflammatory reaction following the injection of tubercle bacilli into the skin was more intense in character, as evidenced by the greater degree of edema and cellular infiltration about the widely dilated vessels. As early as 3 hours after infection, and persisting throughout the period of observation, it was noted that in the syphilitic rabbits the lesions were multiple, focal in character and distributed about the capillaries, whereas in the non-syphilitic rabbits the lesion was single, diffuse in character and bore no relation to the vascular distribution. Histologically the lesions removed at intervals of 48 hours to 3 weeks following the injection of the tubercle bacilli into the syphilitic rabbits consisted of perivascular aggregations of large mononuclear cells, fibroblasts and newly formed capillaries. These lesions resembled in their histological characteristics the primary and secondary lesions of syphilis rather than the characteristic lesion of tuberculosis, although scrapings from more than 200 such lesions examined by means of dark-field have failed to show the presence of *Tr. pallidum*. Epithelioid cells, which were observed in both syphilitic and non-syphilitic rabbits about 2 weeks after infection, were fewer among the syphilitic rabbits and perivascular in their distribution, replacing the large mononuclear cells. On the other hand, among the non-syphilitic rabbits the epithelioid cells appeared in large numbers scattered diffusely throughout the tissue. In the syphilitic rabbits fibroblasts and newly formed capillaries made their appearance within 48 hours after infection with tubercle bacilli and increased rapidly in number, with the formation of dense bundles of connective tissue which separated the lesions, while in the sections removed from the non-syphilitic rabbits there was a paucity of fibroblast formation and an absence of capillaries.

There are considerable data (Bieling,[12] Hektoen,[13] and Clark, Zellmer and Stone [14]) indicating that animals previously immunized to a specific antigen and whose blood serum after a lapse of time contains little or no specific antibody, will, when subsequently injected with an unrelated or remotely related antigen, respond with the production not only of antibodies against the last antigen but also with the production of antibodies against the initial antigen. The antibodies against the initially injected antigen appeared within 24 hours, whereas the antibodies against the second unrelated antigen appeared several weeks later. This revived produc-

tion of antibodies to the original antigen Bieling [12] has termed the "anamnestische Reaktion." That previous or concomitant disease may stimulate the production of antibodies has been noted by Schroeder,[15] who found that rabbits suffering from spontaneous subcutaneous abscesses produced antiserums with a higher titer than did normal rabbits. Similarly, Lewis and Loomis [16] noted that antibody production in tuberculous guinea pigs greatly exceeded that obtained in non-tuberculous animals.

Much less observed data are available to indicate that this same type of "anamnestischem" mechanism is involved in the cellular reaction to an irritant. Tarnowsky [17] and Greenbaum and Madden [18] found that the application of a caustic to the skin of patients suffering from latent syphilis produced characteristic local syphilitic lesions. Klauder [19] and others have shown that syphilitic lesions may occur at the site of trauma, although Klauder was unable to produce an interstitial keratitis in syphilitic rabbits by traumatizing the cornea. Moreover, Greenbaum and Madden could not demonstrate *Tr. pallidum* in local syphilitic lesions produced by trauma.

Our observations on the cellular response of syphilitic rabbits to the injection of virulent bovine type tubercle bacilli suggest that the reaction is of the "anamnestische" character and that the cells of the syphilitic rabbits are so modified that the introduction of an unrelated organism elicits a prompt inflammatory reaction characteristic of the initial syphilitic infection. The perivascular focal character of the lesion, the presence of large mononuclear cells and fibroblasts, and the formation of new vessels suggest syphilis; on the other hand the subsequent appearance of epithelioid cells and of caseation and softening are more characteristic of tuberculosis.

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DESCRIPTION OF PLATES

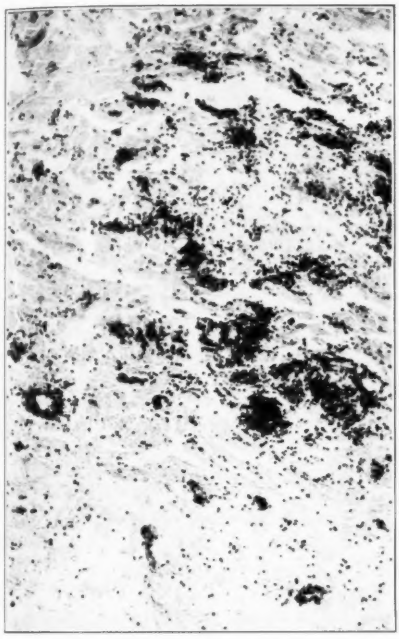
PLATE 35

FIG. 1. Skin of syphilitic rabbit 3 hours after the injection of tubercle bacilli. Edema, aggregations of cells and perivascular infiltration. × 80.

FIG. 2. Skin of non-syphilitic rabbits 6 days after injection of tubercle bacilli. A sharply defined abscess. × 120.

FIG. 3. Skin of syphilitic rabbit 6 days after the injection of tubercle bacilli. Multiple foci of mononuclear cells. × 120.

FIG. 4. Skin of normal rabbit 6 weeks after injection of suspension of *Tr. pallidum*. Focal perivascular aggregation of mononuclear cells. × 400.



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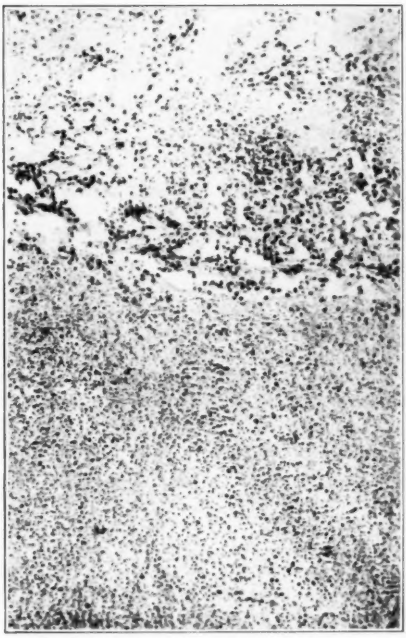

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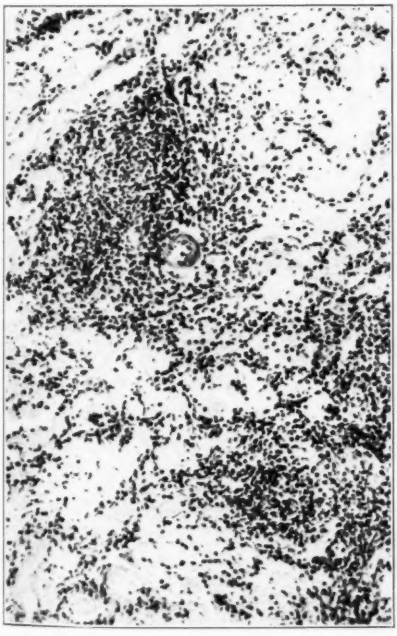

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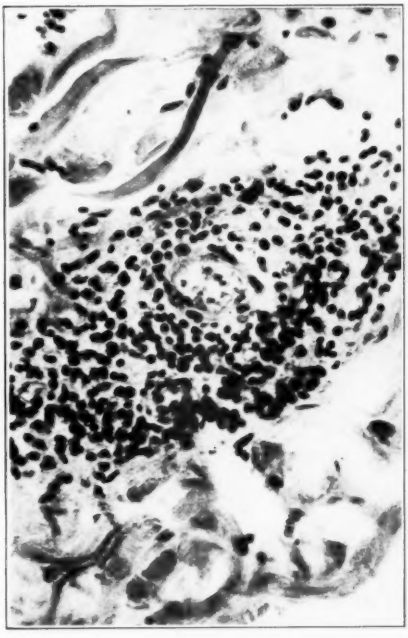

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Effect of Syphilis on Tuberculous Lesions

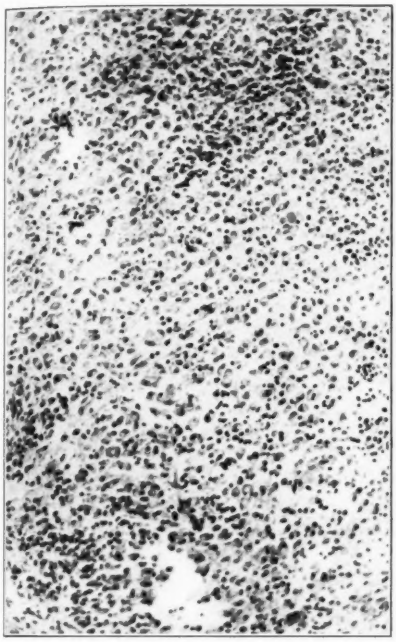
PLATE 36

FIG. 5. Skin of non-syphilitic rabbit 4 weeks after injection of tubercle bacilli. Diffuse infiltration with epithelioid cells. × 150.

FIG. 6. Skin of syphilitic rabbit 4 weeks after injection of tubercle bacilli. Granulation tissue rich in fibrous tissue and mononuclears. × 150.

FIG. 7. Skin of non-syphilitic rabbit 8 weeks after injection of tubercle bacilli. Diffuse infiltration with lymphocytes, plasma cells and epithelioid cells. × 120.

FIG. 8. Skin of syphilitic rabbit 8 weeks after injection of tubercle bacilli. Dense connective tissue replacement. × 120.



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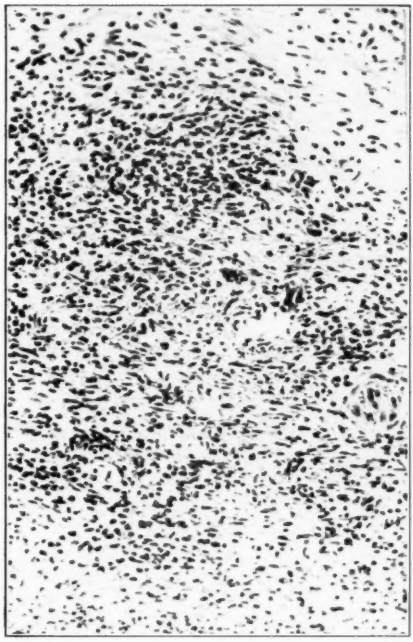

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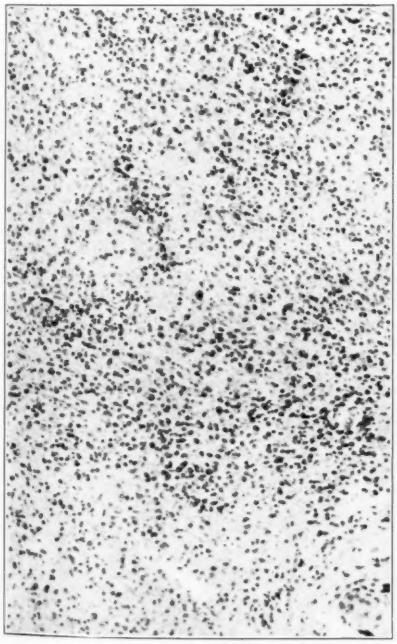

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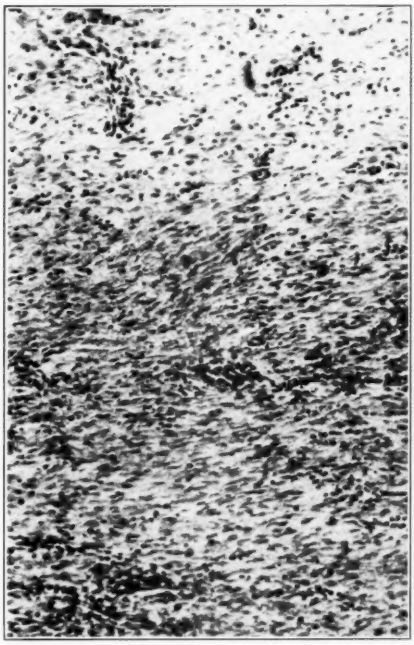

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Effect of Syphilis on Tuberculous Lesions

SPONTANEOUS CARDIOVASCULAR DISEASE IN THE RAT *

I. Lesions of the Heart

S. L. Wilens, M.D., and E. E. Sproul, M.D.

(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, N. Y.)

Relatively little attention has been directed toward naturally developing cardiovascular disease in any species other than man. The contributions of Fox [1] on natural disease in captive wild animals, and of Krause [2] and Wolkoff [3] on the senile changes in the vascular system of domestic animals, serve as outstanding exceptions. Systematic observations of cardiovascular lesions in the common, small laboratory animals have been less often recorded. There are a few sporadic reports such as the description of spontaneous myocarditis in rabbits by Miller,[4] and of medial lesions in the aorta of this same species, originally noted by Ophüls [5] and by Miles.[6]

Even less information is available concerning cardiac diseases to which the rat is naturally susceptible. Löwenthal [7] in 1931 was unable to collect any records of this type of disease in rodents other than those of inflammatory changes in the myocardium. McCay, Crowell and Maynard [8] in a study of the relation of growth to longevity mention that the hearts of old rats are constantly enlarged. A diligent search of the literature has failed to reveal any other reports dealing with this subject although the incidental description of such lesions in control rats used for other purposes may have escaped attention.

The elucidation of this field is of some practical significance. A comparison of spontaneous lesions in animals with those of man may be of value since differences in habits and environment as well as time factors related to life span may shed light on their etiology. It has sometimes been stated (Sherman [9]) that the rat is rather similar to man in its omnivorous food habits and in most aspects of the chemistry of nutrition. It might not be unreasonable to suspect that if these factors play a rôle in the development of

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cardiovascular disease, this species should closely parallel man in this respect.

That distinct specie idiosyncrasies exist in the development of both spontaneous and experimentally produced cardiovascular disease seems fairly well established. Fox [1] describes variations in the types of spontaneous lesions to which different species are vulnerable. Information concerning spontaneous disease may be of importance in evaluating the results of a given experimental procedure. Cowdry and Scott [10] have demonstrated that repeated doses of vitamin D in the monkey do not regularly lead to calcification of arteries, as in the rabbit and rat. Lesions developing in an animal on an experimental regimen are not necessarily the specific result of that procedure even if control animals of a similar age period fail to show them. The procedure may so debilitate and impair the general health of the animal as to precipitate the earlier initiation of lesions that might well have developed spontaneously later in life. The numerous, varied experimental methods that have led to medial degeneration of the rabbit's aorta lose much of their significance when it is appreciated that 87 per cent of all rabbits at 8 months of age exhibit similar lesions (Kesten [11]). The importance of knowing the inherent disturbances and weaknesses of this system in measuring the effects of any experimental procedure is obvious.

For these reasons a systematic study of cardiac disease in a group of 487 rats was undertaken. The animals were derived from an inbred strain of pure albinos of Osborn-Mendel stock and maintained for more than 30 generations in the laboratories of Drs. H. C. Sherman and H. L. Campbell of the Chemistry department of Columbia University.* The influence of diet on longevity in this strain has been previously reported by these authors.[12] From the time of weaning the animals were fed adequate diets of known composition and maintained under constant laboratory conditions until death occurred spontaneously. The group includes 266 females whose average age at death was 746 days, and 221 males averaging 702 days in age at death. The youngest animal to die spontaneously was a female at 79 days, and the oldest also a female at 1124 days. The majority (79.2 per cent) survived longer than

* We wish to acknowledge our indebtedness to Dr. H. C. Sherman and Dr. H. L. Campbell for providing us with the opportunity of autopsying the animals on which this study is based.

600 days and 24 or 4.9 per cent (19 females and 5 males) were older than 1000 days.

The maximum life span of the rat is usually considered to be about 3 years or roughly 1/30th of that of man. At this ratio a period of 100 days in the rat is equivalent to 8.2 years in man. The average age at death in this group of rats would be equal to about 58 years of human life for the male and 61 years for the female. The average age at death for a similar group of humans would compare closely to these figures. The work of McKay, Crowell and Maynard [8] would seem to indicate that the maximum life span in the rat is somewhat longer than 3 years, since they succeeded in sustaining life in 12 out of 106 animals for more than 1200 days. Their oldest rat survived 1421 days. The senile rats of the group reported here showed a high incidence of suppurative infections in the lungs, middle ears, brain and genito-urinary tract. If these had not occurred the survival periods would undoubtedly have been prolonged. Nevertheless, at autopsy even the human subject often shows evidence of infectious processes which may be unrelated to the principal underlying disease. Thus, lobular pneumonia of varying extent is encountered in more than 50 per cent of all human autopsies. It would perhaps be futile to attempt a more accurate comparison of life spans from our present knowledge.

Each animal in the group was subjected to a detailed postmortem examination. Tissues were fixed in Zenker's fluid without addition of acetic acid, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Special connective and elastic tissue stains were employed when required. One complete sagittal anteroposterior section through the entire heart was prepared for microscopic study. This included routinely the left ventricle, interventricular septum, right ventricle, portions of the valve leaflets, auricles and ascending aorta. The following presentation is a description of the pathological findings encountered. The more common lesions are grouped and classified. The incidence and influence of sex and age were determined. The relations of the lesions to each other or to extracardiac changes was explored whenever a possible connection existed. The composition of the diets was analyzed to see if any favored or inhibited the development of these lesions. Reference to diet will be made only when such an influence is indicated.

Endocardium

Intracardiac Thrombosis: In 31 animals (6.4 per cent) thrombi were found in one or more chambers of the heart. 17 of these were females and 14 males. Fairly old rats were particularly susceptible. The average age of the males with cardiac thrombosis at death was 799 days, compared with 702 days for the entire group, and of the females 905 days compared to 746 days for the entire group. In fact, 7 or 29.2 per cent of the 24 animals surviving more than 1000 days showed the lesion. The left auricle was by far the most common site, being involved 20 times. The right auricle was next with 7 cases. Two parietal thrombi had deposited on the wall of the left ventricle overlying areas of myocardial and endocardial fibrosis even though this chamber lacks columnae carneae to form favoring endocardial recesses. Two other thrombi were found loose and detached; one caught in the orifice of the mitral valve, presumably after having broken free from its origin in the left auricle, and one occluding the aortic valve ring.

Only the larger thrombi completely filling the left auricle were recognized grossly. They could be distinguished by their firm inelastic consistence, grayish red color, and by the marked auricular dilatation. The majority of the thrombi in the left auricle were of this type. They apparently obstructed blood flow completely. Usually the thrombi were of recent formation, microscopically showing little or no evidence of organization. They were composed of alternating columns of massed platelets, fragmented leukocytes and intervening deposits of other blood constituents. They were as a rule only loosely attached to the underlying endocardium. In some instances only smaller parietal thrombi were found, most often in pockets of the auricular appendages between musculi pectinati.

Extracardiac infectious processes were neither more severe nor more numerous in this group than in the other animals. Tumor growths were not found in any of the 31 rats. Factors favoring the development of thrombi were usually present in the heart itself. 23 of the animals showed some degree of sclerosis of the coronary arteries, of which 15 were either moderate or severe in degree. 25 showed evidence of myocardial fibrosis, of which 23 were either moderate or severe. As will appear shortly, the incidence of these

two lesions was just as high in animals of a comparable age as in this particular group. Moreover, myocardial fibrosis was rarely encountered in the walls of the auricles. It is therefore difficult to evaluate the rôle of either of these two changes in favoring the formation of intracardiac thrombi.

There was, however, still a third lesion, apparently a chronic inflammation involving chiefly the endocardium of the left auricle, which appeared to play a definite rôle in the precipitation of thrombi in at least some instances by damaging the surface endothelium. Such changes, designated as chronic auriculitis, were found in 8 of the 20 showing thrombi in the left auricle.

Chronic Auriculitis: This lesion was recognized on microscopic examination in 18 (3.7 per cent) rats, 10 females and 8 males. The left auricle alone was involved in 15 cases, twice in conjunction with slight alterations of the left ventricular endocardium and once with minor changes in the right auricle. The lesion attacked a fairly old age group. The average age at death of the females was 819 days, and of the males 758 days. Both sclerosis of the coronary arteries and myocardial fibrosis were extremely common in these animals, but there were 3 in which both of these lesions were entirely absent, so that it can hardly be considered as a secondary extension or complication.

The lesion usually involved the entire endocardial surface of the auricle without extending into the appendage or onto the auricular aspect of the mitral valve leaflets. Microscopically the essential finding was a marked thickening of the endocardial layer. As in man, the left auricular endocardium is normally thicker than the right, although smooth muscle cells cannot be identified. With the development of this lesion, the endocardium increased in thickness 4 to 6 times. The picture varied considerably, depending probably on the stage of development and severity of the process. The endothelial cell lining was usually intact although swollen, basophilic and prominent. Occasionally defects were present and fibrin thrombi were precipitated. The entire endocardium was infiltrated with a variable number of cells. Large numbers of mononuclear wandering cells were frequently encountered, but occasionally lymphocytes, plasma cells or even polymorphonuclear leukocytes predominated. More often there was a mixture of cells including proliferating fibroblasts. The thickening was due in part to the

cellular infiltration but also to an irregular production of connective tissue fibers so that the surface frequently became uneven. In some instances connective tissue proliferation was the conspicuous finding, presumably in arrested or relatively inactive cases. The endocardium did not become vascularized.

The lesion is at once reminiscent of the auriculitis associated with rheumatic heart disease in man, both because of its location and because of its microscopic detail. However, palisading of cells against swollen collagen bands, irregular pyknotic nuclear forms and well defined Aschoff nodules did not occur. In addition, the valve leaflets and perivascular connective tissue of the myocardium were not involved by the specific lesions of rheumatic fever. For these reasons it is impossible to associate it with rheumatic infection. The etiology of the process is completely shrouded although its microscopic characteristics indicate that it is inflammatory in nature. Bacteria were not demonstrable.

Bacterial Endocarditis: The heart valves of the rat were occasionally the seat of bacterial infection which reproduced the local picture of acute bacterial endocarditis in man. 15 such cases (3.08 per cent) are included in this series, 9 in males and 6 in females. The age of these rats at death was widely dispersed, the youngest animal being 138 days and the oldest 1072 days. The average age for the group was well below that of the entire series, being 650 days in the male and 566 days in the female. It is interesting that the incidence in which the individual valves are involved compares closely to that in man.

The mitral valve was attacked 11 times, the tricuspid once, the aortic once, and all three mitral and aortic valves and left auricle once. In one instance the infectious process appeared to originate in the endocardium of the right ventricle, although multiple sections might have revealed a focus in the valve. The microscopic picture was somewhat variable. The less severe lesions centered about the distal ends of the leaflets. More often the entire leaflet was involved, its substance eroded so that only remnants of the valve could be made out. Heavy cellular infiltrations of polymorphonuclear leukocytes and other inflammatory cells occurred regularly. Occasionally abortive attempts at repair were evidenced by accumulations of large mononuclear cells and fibroblastic activity. The damaged surfaces of the valves were covered by bulky

vegetations composed of precipitated fibrin, large bacterial colonies and cellular débris. Often the vegetation filled the entire valve orifice and protruded into the adjacent chambers.

The chief extravalvular finding was metastatic abscesses in the myocardium and kidney, and less often at other sites. The spleen was usually enlarged but not pultaceous. Inasmuch as enlargement of the spleen usually accompanies other infections coexistent in these rats, it is difficult to attribute it solely to the blood stream infection. Gross embolic manifestations were conspicuously absent. In several instances bacteria had gained a foothold in the walls of some of the arteries and had led to arteritis and thrombosis, but infarcts of the spleen and kidney were not seen.

It was not usually possible to establish the exact portal of entry of the bacterial agent. This was due to the presence of too many possible sources rather than too few. Most of the animals had infected ulcerations of the plantar surfaces of the extremities and tail, any one of which might have served as the initial point of infection. In addition, 12 had bilateral suppuration of the auditory bullae, 5 bronchiectatic cavities and abscesses in the lungs, and 2 extensive suppurative endometritis. It must be admitted that infections of the same type and severity were just as frequent in the animals with normal heart valves.

Bacterial cultures were not made so that no accurate information concerning the causative agents can be furnished. Bacteria were, however, readily demonstrable in the vegetations. In most instances they appeared to be Gram-positive cocci, but in two, Gram-negative rods were identified. Although usually the valve leaflets were too completely destroyed to make any positive statements as to their condition previous to infection, there is no reason for assuming that preceding damage existed. Unaffected leaflets in the same hearts showed little or no evidence of alteration.

The normal valve leaflets of the rat are delicate fibrous acellular structures having a little centrally placed elastic tissue and regularly failing to reveal the presence of blood channels. At the base of the aortic valve, cartilaginous plates are embedded so often as to be considered a normal component of the annulus. With advancing age the valves underwent only slight changes. The annulus frequently became larger and often directly continuous with myocardial scars at the base of the heart. Rarely small masses of

calcium were deposited either at the base of the atrioventricular leaflets or in the cartilage of the aortic valve leaflets. The substance of the valve became more solid and compact but remained avascular. Just proximal to the distal end of the mitral valve leaflets on the auricular aspect, and coinciding with what must have been the line of closure, a mound-like eminence of loose edematous connective tissue usually developed. Because of the apparent avascularity of the leaflets, the assumption must be made that bacterial infection occurs as a surface implantation possibly at some minutely damage point on the line of closure. It would probably require technically difficult injection experiments to rule out beyond question the existence of valvular blood channels. Much of the theory that has been expounded to account for the greater frequency of involvement of the mitral valve in man has been based on the more common vascularization of this valve. In the light of the present findings this explanation loses at least some of its validity.

It should be pointed out that in every instance the lesion was that of an acute spreading bacterial infection and never was the picture of subacute bacterial endocarditis in man reproduced. The valvular lesions were too acute and destructive and embolic lesions when they involved the renal glomeruli were frankly purulent.

"*Chronic Endocarditis*": The usual appearance of the heart valves has already been described. In addition to the well defined cases of bacterial endocarditis there were 15 cases in which the heart valves showed minor deviations from the normal. For want of a better term these have been designated as "chronic endocarditis," although the changes were perhaps too minute to be so classified. Never was there obvious deformity or insufficiency of any valve. In 7 the lesions consisted essentially of a mild but definite cellular infiltration of large mononuclear cells into the substance of the mitral valve most conspicuously near the distal end and on the auricular aspect. This was sometimes associated with slight edema of the connective tissue and heaping up of the overlying endothelium. In 4 more, the mitral valve showed, in addition, slight defects in its endothelial surface over which small quantities of fibrin or fibrin-like material were attached. In no instance was there true verruca formation. The tricuspid valve shared these lesions in 3 cases; the mitral and aortic valves were involved together in 1 case. The exact interpretation of these

changes is in doubt. Bacteria were not demonstrable. Possibly they merely represented the results of slightly excessive trauma. The valvular lesions of human rheumatic infection were never very closely simulated.

Myocardium

Fibrosis of Myocardium: One of the most common findings encountered was scarring of the heart muscle. 292 or 59.9 per cent of the animals showed some degree of myocardial fibrosis. Males (65.6 per cent) were more often involved than females (55.3 per cent). The sex difference was more definite when the degree of fibrosis was compared, as in Table I. Here it can be seen that 14.5 per cent of the males and only 6.8 per cent of the females showed severe fibrosis — a ratio of more than 2:1. Fibrosis of the myocardium also occurs more often and severely in the human male than in the female. In the case of the rat, differences in environmental factors cannot be held accountable.

The lesion showed a definite relation to age, tending to involve older animals. The average age at death increased with the severity of the fibrosis in both males and females. In view of the fact that the female rat has a distinct advantage in longevity over the male, the sex difference noted above is even more striking. The average age of the 32 males exhibiting marked fibrosis at death was 805 days and of the 18 females 896 days. The age factor in relation to fibrosis is more apparent from the figures in Table II where the animals are grouped in 100 day intervals. Only 2 males between 500 to 600 days old showed severe fibrosis and none at a younger age. It may be seen that the milder degrees of scarring occurred more often in younger animals, but that animals less than 400 days old almost always had intact myocardia. With advancing age groups, the lesion became more frequent and severe and, in general, the males showed earlier and greater involvement than the female. The impression is gained therefore that fibrosis of the myocardium in the rat depended to a large extent upon age and sex. It appeared to be a progressive lesion having its inception about the 400th day of life and becoming more marked throughout the rest of life. Nevertheless, there was a great deal of individual variation and a good proportion escaped the lesion entirely. Close analogies to human arteriosclerotic heart disease might be drawn,

TABLE I

Incidence of Myocardial Fibrosis

	No fibrosis		Slight fibrosis		Moderate fibrosis		Marked fibrosis		Total with fibrosis	
	No. of animals	Per cent	No. of animals	Per cent	No. of animals	Per cent	No. of animals	Per cent	No. of animals	Per cent
Males	76	34.4	62	28.1	51	23.0	32	14.5	145	65.6
Females	119	44.7	63	23.7	66	24.8	18	6.8	147	55.3
Total	195	40.1	125	25.7	117	24.0	50	10.2	292	59.9

TABLE II

Age Distribution of Myocardial Fibrosis

Age	Sex	No fibrosis		Slight fibrosis		Moderate fibrosis		Marked fibrosis	
		No. of animals	Per cent *	No. of animals	Per cent *	No. of animals	Per cent *	No. of animals	Per cent *
days									
0–400	Male	8	88.9	1	11.1	0	–	0	–
	Female	21	95.5	0	–	1	4.5	0	–
400–500	Male	10	55.6	7	38.9	1	5.6	0	–
	Female	11	100.0	0	–	0	–	0	–
500–600	Male	14	51.9	9	33.3	2	7.4	2	7.4
	Female	9	64.3	4	28.6	1	7.1	0	–
600–700	Male	20	40.0	12	24.0	14	28.0	4	8.0
	Female	15	48.4	11	35.5	4	12.9	1	3.2
700–800	Male	12	21.8	20	36.3	16	29.1	7	12.7
	Female	26	38.8	17	25.4	18	26.9	6	9.0
800–900	Male	9	21.9	10	24.4	7	17.1	15	36.6
	Female	24	36.4	18	27.3	22	33.3	2	3.0
900–1000	Male	2	12.5	3	18.7	9	56.3	2	12.5
	Female	13	36.1	11	30.6	10	27.8	2	5.5
1000–1125	Male	1	20.0	0	–	2	40.0	2	40.0
	Female	0	–	2	10.5	10	52.6	7	36.9

* Per cent of all rats in the same 100 day interval.

not only in chronological relation to life span, but in frequency and sex distribution.

The fibrosis was entirely a microscopic finding. The distribution of the scarring varied somewhat but there were certain vulnerable areas. The left ventricle and interventricular septum were by far the most common sites. The earliest scars were found at the base sometimes extending as tenuous bands from the annulus of the mitral valve or at the tip of the left ventricle. In the latter region the muscle layer is normally quite thin and in advanced cases the entire muscle from epicardium to endocardium was replaced by dense connective tissue. Frequently scars seemed to center about and extend from the adventitia of the larger coronary arteries as they coursed through the muscle layer. At times they were haphazardly distributed. The right ventricle was almost never involved save only at its very base. The auricular muscle remained intact.

For the most part the scarring was partial and rather diffuse, isolated groups of atrophic or hypertrophic muscle bundles being intermingled. Not infrequently fibrous tissue was very finely distributed but widespread, appearing as a generalized increase in loose interstitial connective tissue. Rarely large continuous masses of partly hyalinized, completely acellular connective tissue devoid of muscle fibers had formed. These most closely resembled healed infarcts. True fresh or partly healed infarcts were never found and it seems very doubtful that any of the fibrosis was preceded by necrosis of muscle. Blood pigment and mononuclear cells were usually lacking. The absence of cellular reaction makes it seem most probable that the fibrosis was associated with a slow atrophy of the muscle either due to impaired blood supply or to intrinsic degeneration of the muscle tissue itself. In a few instances cellular reaction was very striking, a variety of leukocytes participating. In these, the evidences of inflammation were so apparent that the lesion might well be classified as chronic myocarditis. This finding occurred so seldom that it is difficult to believe that the more usual type of scarring was the result of a previous inflammatory process which had become arrested. Moreover, the earliest lesions showed no tendency to exhibit an inflammatory character. The specific localizations of the scarring in certain parts of the heart also argues against an infectious background.

As will appear shortly, alterations of the coronary arteries, which would lead to loss of elasticity and hardening if not to complete closure, frequently accompanied the myocardial damage. The weight of the evidence at hand suggests that a process is involved somewhat analogous to arteriosclerotic heart disease in man, namely that with advancing age there is commonly a progressive sclerosis of the coronary arteries interfering with the nutrition of the myocardium and resulting in atrophy and fibrosis. In the rat the vascular lesion is essentially a medial one and never leads to occlusion or thrombosis so that the acute lesions of myocardial infarction do not develop.

Sclerosis of Coronary Arteries: As previously noted, showing close coincidence with fibrosis of the myocardium, the coronary arteries frequently developed changes that consisted essentially of a loss of smooth muscle and a replacement of the media by fibrous tissue. The intima remained thin, the lumen patent, although often reduced to a slit, and the internal elastic lamella although straightened retained its unity. The earliest visible change was a reduction and irregular distribution of smooth muscle nuclei of the media. Some of the muscle cells were hypertrophied and on cross section appeared strikingly vacuolated. Accessory bunches of smooth muscle cells forming imperfect new coats occasionally developed in the adventitia or beneath the intima to give the vessel irregular contours, make it thicker than normal, and encroach somewhat upon the lumen. The adventitia became thickened and densely fibrous. Eventually connective tissue replaced the smooth muscle and this often became hyalinized. Calcification of the media occurred rather infrequently but was noted in 17 cases. It was usually rather scanty, consisting of small deposits in and beneath the elastic lamella. In one instance the calcification was extensive enough to form curved plates within the media, incompletely encircling the entire circumference of the right coronary artery just beyond its point of origin. The intima remained delicate, consisting of a single layer of endothelium closely approximating the subjacent elastic fiber. Lipoid deposits in the intima, similar to atherosclerosis of man, were not observed.

The coronary arteries of the rat's heart are two in number and arise from the sinuses of Valsalva at points similar to the origin of the coronary arteries in man. They also follow the usual distribu-

tion of the human vessels, the right being slightly the larger and supplying portions of the left ventricle posteriorly and the interventricular septum. In the atrioventricular sulci the coronary arteries lie in the subepicardium sometimes embedded in a slight amount of adipose tissue. Subepicardial fat in the rat's heart is always scanty, even in well nourished animals, and is confined to the base of the ventricles. All the branches of the main coronary arteries equivalent to the descending branches in man are definitely intramuscular, although the coronary veins course superficially. The microscopic appearance of the normal coronary artery shows no striking peculiarities. They possess a single internally placed, wavy elastic lamella. Both vessels appeared to be involved with equal frequency and severity by the sclerotic process. The most profound changes were found in the main arteries just beyond their points of origin but the lesion extended well into the intramuscular branches. One exception was a small artery rather constant in position near the base of the anterior leaflet of the mitral valve which often showed marked fibrosis when all other vessels were quite normal.

From Table III it may be seen that, as in fibrosis of the myocardium, the male is more often and severely affected than the female. The incidence of severe coronary sclerosis is again almost 2:1 in favor of the male. Similarly it may be seen that the total incidence of each grade of involvement closely parallels that of myocardial fibrosis by comparing the figures in Table I. 57.7 per cent of all the animals showed some degree of coronary arteriosclerosis, whereas 59.9 per cent showed some myocardial fibrosis.

Table IV shows the age distribution at 100 day intervals and discloses that age plays an analogous rôle in the development of the arterial lesion, as it did in myocardial fibrosis. There is this exception however. The milder degrees of arterial change not infrequently made their appearance in the first 400 days of life. This might be taken as evidence that the vascular lesion antedated the muscular scarring and if any cause and effect relation between the two lesions existed, the arteriosclerosis was responsible for the subsequent myocardial degeneration.

Although the two lesions were closely linked as regards frequency, age and sex distribution, the two did not always parallel each other in individual cases. For example, 16 animals that

TABLE III

Incidence of Sclerosis of Coronary Arteries

	No sclerosis		Slight sclerosis		Moderate sclerosis		Marked sclerosis		Total with sclerosis	
	No. of animals	Per cent	No. of animals	Per cent	No. of animals	Per cent	No. of animals	Per cent	No. of animals	Per cent
Males	86	38.9	51	23.1	59	26.7	25	11.3	135	61.1
Females	120	45.1	72	27.1	58	21.8	16	6.0	146	54.9
Total	206	42.3	123	25.3	117	24.0	41	8.4	281	57.7

TABLE IV

Age Distribution of Sclerosis of Coronary Arteries

Age	No sclerosis		Slight sclerosis		Moderate sclerosis		Marked sclerosis	
	No. of animals	Per cent	No. of animals	Per cent	No. of animals	Per cent	No. of animals	Per cent
days								
0–400	25	80.6	4	12.9	2	6.5	0	–
400–500	22	75.9	3	10.3	4	13.8	0	–
500–600	23	56.1	13	31.7	5	12.2	0	–
600–700	41	50.6	20	24.7	13	16.1	7	8.6
700–800	46	37.7	35	28.7	28	23.0	13	10.6
800–900	30	28.0	27	25.2	37	34.6	13	12.2
900–1000	19	38.5	11	21.2	17	32.7	5	9.6
1000–1125	0	–	10	41.7	11	45.8	3	12.5

exhibited moderate or marked fibrosis of the myocardium revealed apparently normal coronary arteries. On the other hand, 10 animals showing moderate or severe sclerosis of the coronary arteries had intact myocardia. It is likely that a more extensive microscopic examination would have yielded a closer correlation in these exceptional cases. Reference to Table V will show that in general there is a fairly close correspondence between the two lesions. 77.5 per cent of the animals showing no myocardial fibrosis had normal coronary arteries and 84 per cent with either moderate or severe fibrosis also had either moderate or severe sclerosis of the coronary arteries. In arteriosclerotic heart disease in man, the severity of the coronary artery lesion usually parallels the degree of myocardial damage but individual cases show just as marked discrepancies as do the two lesions in the rat.

Coincidental extracardiac lesions were analyzed to see if they played a rôle in the development of these lesions but no such relationship was discovered. One lesion of interest in this connection is the pulmonary one consisting of a combination of bronchiectasis and lung abscess and occurring in varying degree in about 75 per cent of the animals. Both by reason of its high incidence and because of the effects of a badly impeded pulmonary circulation, it is conceivable that this lesion might have been a factor in producing cardiac changes. However, distinct hypertrophy of the right ventricle was lacking. Moreover, 26 per cent of the animals showing little or no bronchiectasis had moderate or severe cardiac lesions. On the other hand, 31 per cent showing severe or moderate bronchiectasis had little or no cardiac damage, whereas only 19 per cent with equally severe bronchiectasis exhibited severe or moderate cardiac lesions. The implication of the latter finding is that animals with marked pulmonary lesions may have succumbed before reaching the age in which severe lesions of the heart were prevalent. Furthermore, the pulmonary lesions did not progressively increase in frequency with advancing age so uniformly as the cardiac ones. For these several reasons it seems quite unlikely that the cardiac damage resulted secondarily from impaired pulmonary circulation.

Cardiac Hypertrophy: The size of the heart of the adult rat at spontaneous death varied considerably and was dependent largely on the body size of the animal and the degree of dilatation. Since

TABLE V

Relation of Myocardial Fibrosis to Sclerosis of Coronary Arteries

	No sclerosis		Slight sclerosis		Moderate sclerosis		Marked sclerosis	
	No. of animals	Per cent	No. of animals	Per cent	No. of animals	Per cent	No. of animals	Per cent
No fibrosis	151	77.5	34	17.5	8	4.1	2	1.0
Slight fibrosis	39	31.2	53	42.4	26	20.8	7	5.6
Moderate fibrosis	13	11.1	31	26.5	63	53.9	10	8.5
Marked fibrosis	3	6.0	5	10.0	20	40.0	22	44.0

the weight of the animal was influenced by the state of nutrition and the amount of postmortem dehydration, the ratio of body weight to heart weight was not a reliable criterion of hypertrophy. The weight of the heart was not recorded and estimations of hypertrophy were based on total size, ventricle wall thickness and the microscopic diameters of the individual fibers. It is admitted that these criteria are largely subjective and liable to error. 173 rats, 86 males and 87 females, were considered to have definite hypertrophy of the heart in some degree, an incidence of 35.5 per cent. Although the sexes were about equally involved, the enlargement in the male was often more striking. Hypertrophy was rarely detectable before 500 days of age and became more frequent with advancing age thereafter. The left ventricle was the chief site, so that this chamber often occupied a disproportionately large part of the entire organ. The increase in muscle was usually associated with some degree of dilatation.

Microscopically the muscle bundles were increased in diameter. This was more striking at the base of the left ventricle rather than at the apex, and often hypertrophic fibers were unevenly distributed, leaving many groups of muscle bundles unchanged. The structure of the hypertrophic fiber was essentially unaltered. The nuclei were not strikingly enlarged but were irregular and hyperchromatic. The interfibrillar substance was not increased.

Many of this group showed a coincidental myocardial fibrosis and changes in the coronary arteries. This is not surprising when it is recalled that similar age groups are involved. The total incidence of hypertrophy was much lower than that of the other two lesions, so that it was not an inevitable accompaniment of the latter. In fact, many of the most severely fibrotic hearts were quite small. 43 per cent of the animals with no hypertrophy did have some degree of fibrosis. Hypertrophy without fibrosis was seen in only 14, about 8 per cent of all those without fibrosis. The inference may be drawn that hypertrophy often accompanied fibrosis and coronary arteriosclerosis but was by no means a constant concomitant. Hypertrophy in the absence of other myocardial changes, although uncommon, also occurred. As in man, enlargement of the heart not infrequently accompanied chronic renal disease. 24 per cent of the hypertrophy group also had relatively severe renal lesions.

Infrequent Myocardial Lesions: Suppurative infections were extremely common in the entire series. The most usual sites were the auditory bullae, lungs, uterus, cranial cavity, kidneys, prostate and liver. In a few instances bacteria had gained entrance into the blood stream and given rise to metastatic foci. The heart was one of the most common sites for such secondary abscesses, being involved 17 times. The abscesses were usually small, fresh, well demarcated, often multiple and without an encapsulating membrane. They consisted of disintegrating polymorphonuclear leukocytes and centrally placed clumps of bacteria.

Aside from these definite foci of infection, it was not unusual to find a few scattered cells infiltrated into the interstitium and often in the adventitia of blood vessels. Occasionally these were grouped together in submiliary collections so as to resemble somewhat the appearance of Aschoff nodules, although in no instance was the reproduction very exact. Such lesions did not occur in conjunction with endocardial or pericardial changes. These cellular aggregates exhibited much individual variation. In 21 cases the resemblance to human Aschoff bodies was fairly close. They were apt to occur in hearts that were considerably scarred but could also be found in otherwise normal areas. Occasionally the cells in them were large basophilic and had hyperchromatic, heavily rimmed nuclei with prominent nucleoli. Swollen fragmented collagen bundles were usually lacking and never very definite.

The series includes 17 cases of leukemia in which infiltrations of leukemic cells into various organs were common. The heart was infrequently invaded but twice there were small collections of leukemic cells penetrating the interstitial and subendocardial tissue.

Relatively few malignant growths were observed in the present series and most of these were of connective tissue origin rather than epithelial. Only one, an osteosarcoma of undetermined origin had metastasized to the heart.

Pericardium

Acute Suppurative Pericarditis: The pericardial sac was infected with bacteria 11 times, an incidence of 2.3 per cent. Five times the resultant inflammation was confined to small areas, usually at the base of the heart over the auricular epicardium, but in the others the entire surface was covered by thick layers of fibrinopurulent

exudate. Bacteria were readily demonstrable. Granulation tissue was seen at times in the deeper layers of the exudate. In 9 of these 11 there was extensive pulmonary and pleural infection, and in several direct extension to the pericardium could be traced. The remaining 2 had suppurative lesions elsewhere which might have served as an initial source. The underlying myocardium and the endocardium were unaltered.

Acute pericarditis in rodents caused by infection with a streptothrix has been described by Berberich and Nussbaum [13] in hemorrhagic septicemia, and with a diplococcus by Seifried.[14] The pericardial infection was the result of extension from pulmonary foci.

Chronic Pericarditis: The pericardium of the rat appears to be subject to a specific inflammatory process which because of its distinctive histological appearance seems to be a disease entity. The picture was that of a low grade, persistent inflammation attended by the infiltration of lymphocytes, plasma cells, large mononuclears and the local proliferation of mesothelial cells and fibroblasts. The epicardium was thickened and densely cellular. The overlying mesothelium was swollen, basophilic, heaped up in villus protrusions and even stratified. Often the infiltrating cells were lined up in palisade formation parallel to the surface and lodged against long acellular bands of eosinophilic collagen. Such palisades were often multiple. In more severe cases the surface mesothelium was disrupted and minute quantities of a fibrinoid substance were deposited in the exposed surface. Occasionally eosinophils were fairly numerous but neutrophilic polymorphonuclear leukocytes were always infrequent.

The process was not uniformly distributed and was usually most advanced in crevices and sulci about the auricular appendages or in the atrioventricular groove. Less often it involved the entire epicardium, but unevenly, with mound-like eminences irregularly scattered. The normal parietal pericardium was apparently so delicate as to be invariably ruptured on removing the chest plate. At any rate it was not often detected. Pericardial adhesions, which from the nature of the lesion might be reasonably expected, were not encountered. Grossly the epicardial surface was described as smooth but milky gray and translucent.

The lesion was not confined to the percardium but frequently involved both pleural cavities and the peritoneum. In the latter

it was most often seen over the capsules of the spleen and liver, around the pancreas, mesentery and posterior peritoneal surface. The microscopic appearance was always essentially the same. In neither pleura nor peritoneum did obliterative adhesions form. Aside from the serous surfaces, no other tissues were involved. The process may be considered to be essentially a polyserositis.

29 such cases were recognized, an incidence of 6 per cent. In 13 all three serous cavities were involved and in the remainder the pericardium alone was attacked. One striking feature was its decided preponderance in female rats, there being only 5 males to 24 females in the group. The lesion was found in comparatively old rats. The age at death of the females was 878 days and of the males 735 days.

The etiology of this process is completely obscure. Microorganisms were not demonstrated, but cultures were not taken. Intracellular inclusion bodies were not encountered. It bore no definite relation to any other disease process. Suppurative infections, although common, were not more so than in the other animals. Three of the rats had inflammatory changes in the left auricular endocardium previously designated as auriculitis. Since both lesions were relatively infrequent (6 per cent and 3.7 per cent), this association is perhaps higher than might be expected. Myocardial fibrosis and cardiac hypertrophy were not more marked than in animals of similar age periods. Because of its distribution and chronicity, one is reminded of Pick's disease in humans, but arrested cases with calcification, hyalinization and obliterating adhesions were never observed. Occasionally several cubic centimeters of clear pale fluid were contained within the peritoneal cavity, but larger accumulations were lacking.

A similar disease, possibly etiologically related, has been described in guinea pigs by Steinmetz and Lerche [15] and more recently by Roth.[16] A Gram-labile bacillus was recovered from the lesions but an etiological relationship has not been definitely established. The guinea pig disease differs from that in the rat in that it is acute and fatal.

Summary

The pathological manifestations of cardiac disease in a group of 487 inbred albino rats maintained on adequate diets and under

constant laboratory conditions over their entire life span are described. The animals were not subjected to experimental manipulation and the disorders encountered must be considered to have evolved from spontaneous causes and under natural circumstances. The rat's heart proved to be quite susceptible to a variety of disease processes, some of which are distinctive and peculiar to this species, while others have their counterpart in man. A simple tabulation of each type of lesions described and its incidence follows:

Endocardium

Intracardiac thrombosis	6.4%
Chronic auriculitis	3.7%
Acute bacterial endocarditis	3.4%
"Chronic endocarditis" (without valvular insufficiency)	3.4%

Myocardium

Fibrosis of myocardium	59.9%
Sclerosis of coronary arteries	57.7%
Sclerosis of coronary arteries with calcification	3.7%
Hypertrophy of myocardium	35.5%
Abscess of myocardium	3.7%
"Interstitial myocarditis"	4.3%
Leukemic infiltration	0.4%
Secondary osteosarcoma	0.2%

Pericardium

Acute suppurative pericarditis	2.3%
Chronic pericarditis	6.0%

Both the lesions classified as chronic auriculitis and chronic pericarditis are apparently peculiar to the rat, although the latter lesion may be related to a similar condition occurring in guinea pigs. They consist essentially of long-standing, low grade inflammatory changes. In neither case was the responsible etiological agent identified. The pericardial lesion appears to be merely one expression of a generalized disturbance of the serous surfaces.

All the other processes described resemble in part at least those recognized in man. The acute bacterial infections certainly differed in no essential manner. Intracardiac thrombosis, chiefly of the left auricle, is somewhat similar to the auricular appendage thrombosis in the diseased human heart. In the rat, however, the thrombus usually filled the entire auricle and occurred as a terminal event, especially in senile animals. Myocardial fibrosis of the left

ventricle is common to both species but in the rat it is less obviously the result of reduced arterial circulation. Changes in the coronary arteries are common and do parallel the myocardial lesions but they never lead to complete or even marked occlusion.

With the exception of the infectious processes, almost all of the changes described make their appearance late in the 2nd year of life and do not attain their maximum incidence until well into the 3rd year. These periods correspond roughly to late middle age and early senescence. Many of the human cardiac conditions have a similar age distribution. In both species the male is somewhat more susceptible to this type of disease than the female.

Notable in the rat by their absence are the intimal atheromas of human coronary artery disease, evidences of myocardial infarction, chronic valvular deformities and rheumatic infection. Slight, apparently non-specific inflammatory changes of the mitral valve and perivascular tissue of the myocardium, which might be erroneously construed if encountered in experimental animals, do occur but are relatively rare.

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DESCRIPTION OF PLATES

PLATE 37

FIG. 1. Acute bacterial endocarditis and acute suppurative pericarditis. The arrow indicates a ball-like vegetation on the mitral valve leaflets. The epicardium is everywhere coated by a thick layer of fibrinopurulent exudate.

FIG. 2. Cardiac hypertrophy. The variation in size of the heart at death in 2 old rats is indicated. The one on the left is considerably hypertrophied.

FIG. 3. Thrombosis of auricle. The left auricle is completely obstructed by a thrombus loosely united to the underlying intact endocardium. The thrombus still shows distinct platelet columns but the leukocytes at their margins are disintegrating. × 60.

FIG. 4. Chronic auriculitis. The endocardium of the left auricle is irregularly thickened by a dense cellular infiltration of polymorphonuclear leukocytes, lymphocytes and large mononuclear cells. The surface endothelium is swollen. × 460.

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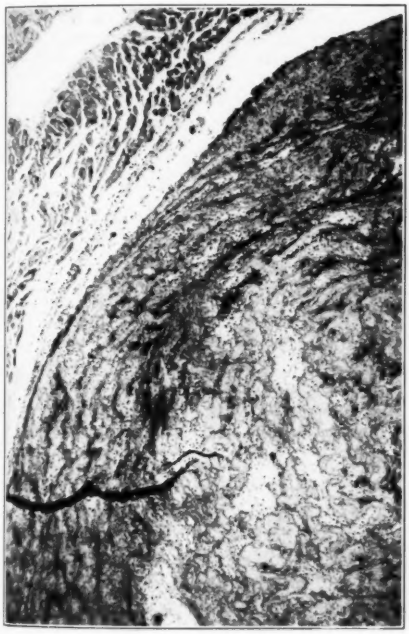

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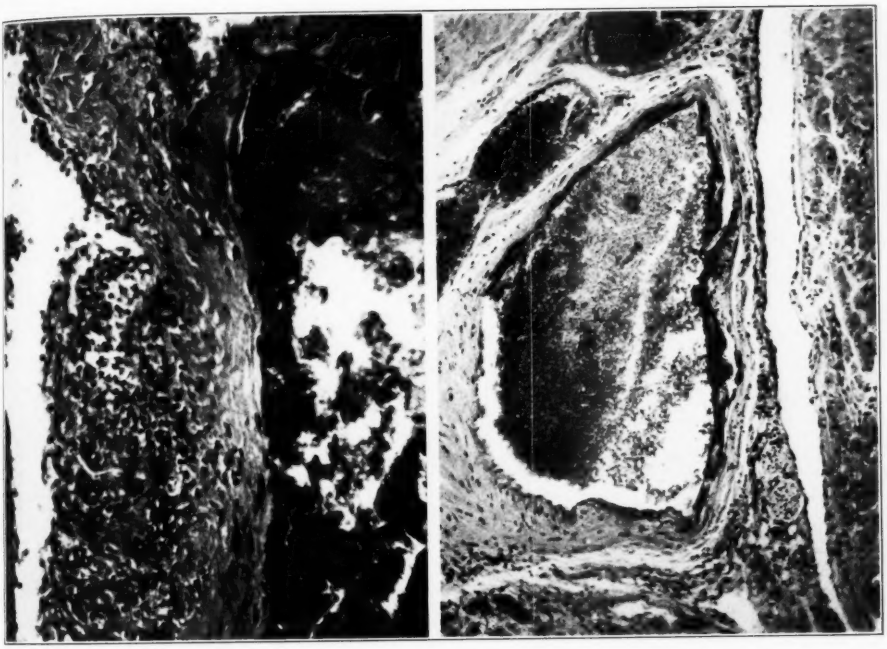
PLATE 38

FIG. 5. Acute bacterial endocarditis. The remains of the mitral valve leaflet are heavily infiltrated with polymorphonuclear leukocytes. The surface is ulcerated and on one aspect a vegetation containing small, deeply staining bacterial colonies is deposited. × 110.

FIG. 6. Calcification of coronary artery. The calcium is in the form of a narrow but continuous band extending around most of the circumference of the vessel. The deposit is confined to the intima and inner aspects of the media. × 60.

FIG. 7. Sclerosis of coronary artery. The lumen is reduced to an elliptical slit lined by intact endothelium. The media is converted into dense acellular collagen. A few strikingly vacuolated smooth muscle cells persist in its outer layers. The adventitia is also thickened by fibrous tissue. × 460.



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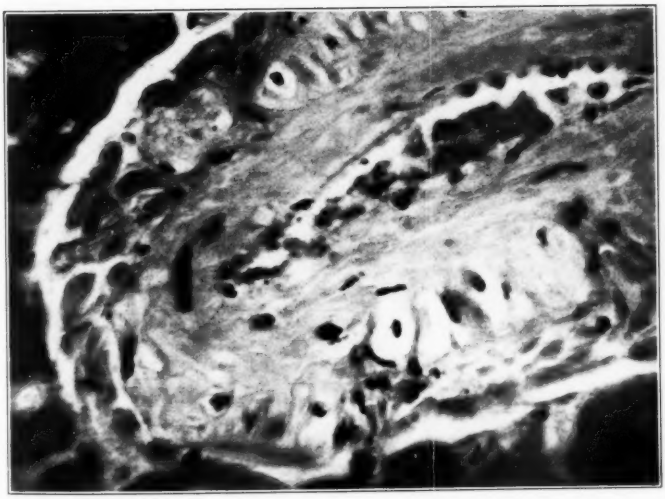

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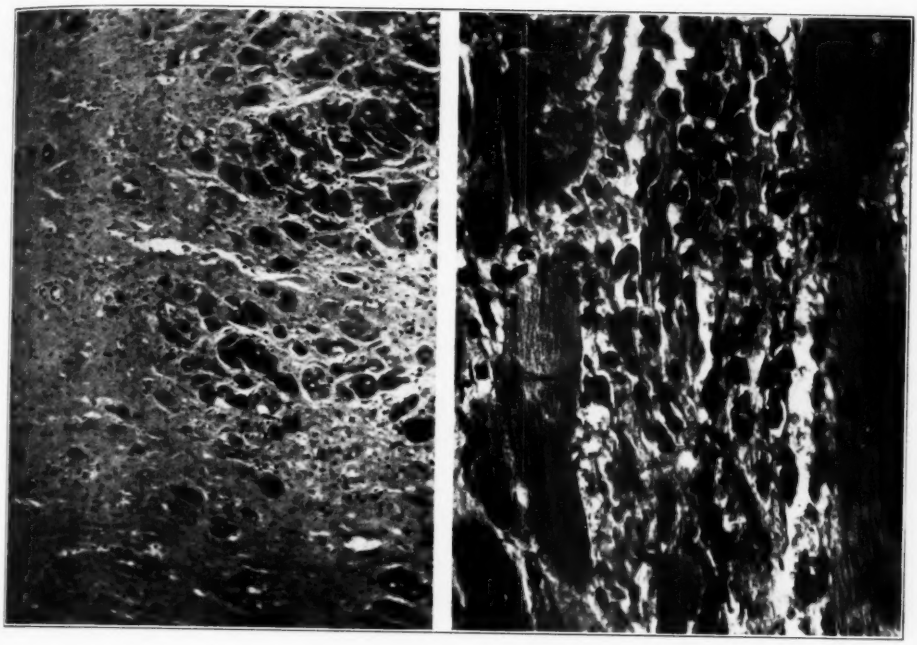
PLATE 39

FIG. 8. Fibrosis of myocardium. The margin of a large, dense, relatively acellular scar is shown where it adjoins and extends between atrophic and hypertrophic muscle bundles. × 110.

FIG. 9. "Interstitial myocarditis." A focal area of cellular infiltration separates adjacent muscle bundles. Lymphocytes, large mononuclear cells and fibroblasts are irregularly dispersed between collagen fibers. × 460.

FIG. 10. Chronic pericarditis. The epicardium is thickened, vascularized and densely infiltrated with a variety of leukocytes. A slight amount of fibrin is deposited at the surface. × 300.

FIG. 11. "Chronic endocarditis." The section is through an area of edematous thickening near the distal extremity of the leaflet. On the auricular aspect there are villus-like irregularities filled with pyknotic and fragmented nuclei. Beyond this point within the leaflet the collagen is swollen, granular, disorganized and deeply eosinophilic. × 300.



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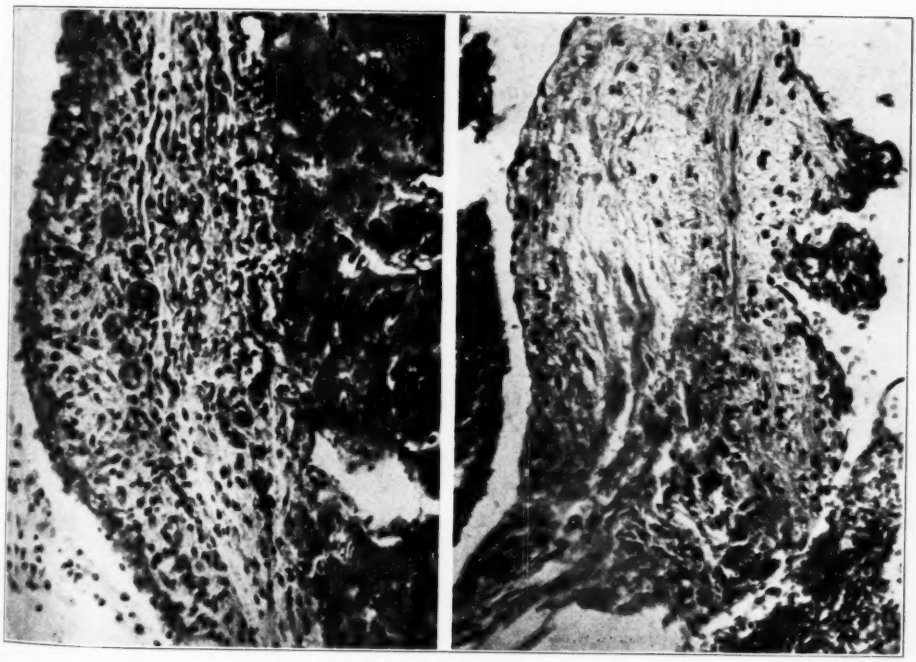

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Wilens and Sproul Spontaneous Cardiovascular Disease. I

SPONTANEOUS CARDIOVASCULAR DISEASE IN THE RAT *

II. Lesions of the Vascular System

S. L. Wilens, M.D., and E. E. Sproul, M.D.

(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, N. Y.)

In a previous article [1] on this subject it was pointed out that information concerning spontaneous vascular disease in the rat is inadequate and incomplete. Duff [2] in a recent review lamented the fact that so little is known concerning natural vascular disease in common laboratory animals. Because of this lack, interpretation of the results of experimentally induced vascular changes is rendered uncertain. Most of the general articles on vascular disease in animals have omitted reference to the rat. Wolkoff [3] included studies on the arteries of 3 rats in different age periods, the oldest of which was 2 years. She was unable to find any very definite changes in the intima or elastica other than slight splitting of the latter in the abdominal aorta of 1 animal. Hueper [4] reported briefly on the incidental finding of changes in the pulmonary arteries associated with calcification in 12 of 75 adult rats.

Spontaneous intimal atheromatous lesions similar to those of man are apparently of limited occurrence in any other species except birds. Minimal and somewhat questionable lesions of this type have been described however in a few mammals, including monkeys and dogs (Fox [5] and others). Löwenthal [6] has reported impregnation by lipoid of arterial walls in several mice but no definite intimal plaques. Nevertheless the absence of such lesions in the rat has never been conclusively established.

Some of the problems of senescence are best approached by the study of animals such as the rat whose natural life span is relatively brief. Alterations of the vascular system, particularly of its elastic tissue component as an indicator of old age have attained almost proverbial acceptance. However, it has always been difficult to distinguish those changes that are the inevitable result of aging from the consequences of disease processes. Lesions that are common to senile animals of many species are more apt to be the

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true expression of senescence than those that are peculiar to only a few. How much such changes depend on simple time factors and how much upon the physiological status of the animal remains unknown. In man with a maximum life span of about 90 years it takes many decades for evidences of senescence to appear. It would be interesting to know if the tempo of these changes is accelerated in animals with short life periods, such as the rat, so as to reproduce a picture similar to senility in man. Obviously if this were so, senescence would depend less upon the simple aging of tissues and more upon intrinsic, less readily explainable phenomena directly related to the natural life span of each species.

In an attempt to shed light on some of these problems a systematic study of vascular changes in a group of 487 rats kept under constant conditions over their entire natural life span was undertaken. The source and nature of this material as well as the vital statistics concerned have already been detailed.[1] Descriptions of the lesions affecting the coronary arteries were included in this earlier report on cardiac disease in the rat. In the present study the vessels of the lungs, spleen, liver, pancreas, adrenals, kidneys, pelvic organs, stomach, neck organs including thyroid and parathyroid, and brain were routinely studied in every case by means of single microscopic sections through each of the organs enumerated. Portions of the ascending aorta were included in every heart section. In addition, in many instances the entire aorta was sectioned. The blood vessels in other tissues such as the testes, intestine, mesentery, pituitary, bone and bone marrow were also frequently examined. Those of the extremities were not investigated.

Aorta

The normal rat's aorta is a delicate, thin walled tubular structure only slightly thicker and larger at its root than at the bifurcation. Microscopically the media contains a series of parallel, slightly wavy elastic fibers varying in number from 8 to 14. The individual elastic fibers are approximately as thick as those in the media of the human aorta but they are never quite so sinuous even in young animals. The elastic fibers are separated from each other by a double layer of smooth muscle cells so that they never overlap in an entangling fashion and the course of each fiber is readily

traced. At infrequent intervals short connecting elastic fibers branch off at acute angles from the main fiber to extend between the smooth muscle cells and anastomose with immediately adjacent ones. The proportion of elastic tissue to smooth muscle is much less than in the human aorta. Connective tissue is scanty and the reticulum is delicate but forms a uniform pattern as it ensheaths the coarser elastic fibers. The most internally situated elastic fiber is separated from the overlying endothelium of the intima only by delicate reticulum. The intima therefore consists of little more than the surface endothelium and its basement membrane. The adventitia is delicate and is composed of acellular loose connective tissue. Nutrient vessels never penetrate the media even at its outer aspect and indeed are not seen in the adventitia itself throughout the entire length.

With advancing age the aorta developed only relatively mild changes. It was somewhat increased in length and circumference. The adventitia was thickened and its connective tissue more compactly arranged. The media was also considerably thicker but its architecture was essentially unaltered. The intima remained as delicate as in young animals. The only lesion of note was the rare occurrence of small masses of calcium usually in the inner third, sometimes protruding into the lumen through disrupted elastic lamellae although always surmounted by intact endothelium. Such calcification was observed in 12 aortas from 9 males and 3 females. All except 1 of these were older than 700 days. The most common sites were in the lower abdominal region, near the bifurcation, at the upper limits of the sinuses of Valsalva and at the angles formed by the orifices of large branches. The calcified masses rarely exceeded 100 μ in diameter and never were more than three such deposits found in any one aorta.

One of the few invariable consequences of aging in man is alterations in the elastic tissue, chiefly a straightening and loss of waviness of the individual fibers. This is associated with enlargement of the vessels and loss of inert elasticity. It has been shown [7] that this change is directly proportional to age and is not affected by intimal atheromas. For this reason the pattern of the elastica of young and old rats was compared to see if analogous changes occurred in the 3 year period that constitutes the life span of this species. Obviously if at the end of this period the rat has under-

gone changes comparable to senility in man one might well expect its elastic tissue to show evidences of degeneration.

Such a comparison offers difficulties for two reasons. First, the elastic fibers of young rats are not so coiled as in the young human aorta, possibly because in the former the aorta does not undergo as relatively great an excursion with each pulsation. Secondly, the relatively large proportion of smooth muscle tissue in the rat's aorta may serve to hold the elastic fibers in unnatural positions after death. The degree of contraction and postmortem rigor would thus play a more important rôle in the rat than in the human in determining the appearance of the elastic fibers. Nevertheless, examination of a large number of preparations stained by Weigert's elastic tissue method revealed mild but constant losses in waviness of the elastic fibers in old rats, as compared to those of young ones. If this change be considered an expression of senescence, then a 3 year old rat is in a physiological state comparable to that of an old man, at least as far as its aortic elastica is concerned. Moreover, the changes in these fibers are not necessarily the result of simple physico-chemical reactions occurring at fixed time intervals but are dependent upon natural life span of the species.

The Arteries

In general, the arterial system of the rat developed no such constant morphological changes with increasing age as that of man. The only two exceptions were the coronary and pulmonary arteries. All other vessels, including the renal and cerebral arteries, usually retained the same characteristics in the senile rat as in the adolescent one. The intima remained thin and delicate. The elastica did not become frayed or reduplicated. The smooth muscle of the media was essentially unaltered. The vessels increased slightly in size but were never stiffened or tortuous.

The failure to form definite lipoid-containing atheromas in the intima was striking when contrasted with the usual findings in man. This did not appear to be associated with inability to deposit cholesterol in other sites. Indeed it was a common occurrence to find considerable quantities of cholesterol free, in crystalline form, and finely divided in droplets within phagocytes in areas of old inflammation. This was particularly true in the walls of old pul-

monary and uterine abscesses. Occasionally, even in otherwise normal lung tissue, there were found groups of subpleural alveoli filled with fat-laden phagocytes. The failure of atheromas to develop would therefore seem to depend less upon the inability to mobilize and deposit cholesterol than upon local factors obtaining within the arterial system. Special lipoid stains were not prepared routinely so that it is impossible to exclude the possibility of subintimal impregnations by lipoid, such as were described by Löwenthal [6] in a number of mice. Some of the mouse deposits were associated with inflammatory changes of the vessel walls.

TABLE I

Incidence, Age and Sex Distribution of Calcification of Arteries

Artery	No.	Per cent	No. of males	No. of females	Average age	
					Males	Females
					days	*days*
Spermatic	49 *	58.4	–	–	745	–
Pulmonary	224	46.0	114	110	724	768
Coronary	17	3.5	14	3	756	850
Aorta	12	2.5	9	3	803	911
Renal	8	1.6	8	0	754	–
Mesenteric	6	1.2	3	3	843	864
Cerebral	2	0.4	2	0	792	–

* The testes of only 83 males were examined microscopically.

Calcification of Arteries: Although generalized manifestations of degeneration were usually lacking, the one most common pathological alteration observed was calcification. In order of frequency calcification was noted in the spermatic, pulmonary, coronary, renal, mesenteric and cerebral arteries, as well as in the aorta itself. The calcium was usually deposited in small solid masses. The earliest portions of the vessel involved were the inner layers of the media, sometimes with impregnation of the elastic lamella alone. Larger deposits extended throughout the media and protruded through the intima into the lumen. The calcification was never very extensive and seldom did more than 3 arteries in any 1 animal have such deposits. In Table I the incidence, age and sex distribution of the findings are recorded. Males showed arterial

calcification more frequently than females. The average age for each group exceeded that of the entire series which was 702 days for males and 746 days for females.

The intratesticular branches of the spermatic artery were calcified in 49 of 83 testes that were examined microscopically. Not only was this the most frequent site but the extent of calcification was greater than elsewhere. The calcification was confined to the media which was frequently converted into a continuous ring of calcium. The deposits occurred in vessels that were otherwise normal, the intima remaining intact and delicate. They were seldom detected in animals less than 500 days old at death. The adjacent tubules did not show changes comparable to those of the senile testis in man. The basement membranes were thin, spermatogenic epithelium abundant, and interstitial cells not increased. Next in frequency of calcification were the pulmonary arteries. Here the process appears to be definitely related to sclerotic changes in the vessel wall and will therefore be described separately in detail. Identical lesions have been briefly described in 12 out of 75 adult rats by Hueper.[4]

Pulmonary Vascular System: The pulmonary arteries of the rat were peculiarly susceptible to degenerative changes. These were not unlike the ones involving the coronary arteries but were often more widespread and severe. The essential lesion consisted of atrophy of the smooth muscle coat and replacement by fibrous tissue leading to irregular thickening of the wall. In less involved areas the smooth muscle often appeared hyperplastic. The chief differences from the coronary artery lesions were that they were found in almost every rat over 2 years of age and were commonly associated with calcium deposition. The lesions developed at all points in the course of the vessels and were even continuous throughout to the smallest arteriolar radicles. Usually, however, the proximal supravalvular portions were not conspicuously involved.

The pulmonary veins were unchanged but they did exhibit an anatomical peculiarity already described in the literature by Lauche [8] and by Takino.[9] The outer aspect of the pulmonary veins consists of several layers of cardiac muscle bundles having all the characteristics of ordinary heart muscle. Sections through the pulmonary veins at their entrance into the heart show that this

muscle is directly continuous with that of the left auricle. Even deeply within the pulmonary tissue the smaller veins have an outer coat of cardiac muscle. This is separated internally from a quite thin and uneven layer of smooth muscle by loose connective tissue. The significance of the extra coat of aberrant cardiac muscle is not apparent but perhaps cardiac impulses are directly transmitted to the pulmonary circulation.

Direct connection between the deposition of calcium and the severity of local sclerotic changes in the artery wall could not always be established. Often calcium masses were found in vessels that were otherwise normal. More often still such changes as could be recognized in the adjacent vessel wall might well have developed subsequent to calcification. The calcium showed a striking tendency to deposit at points of bifurcation or in the angle formed by the origin of a large branch. Another prominent feature was the projection of the calcium as jagged spurs into the lumen and over which no endothelial covering was detected. In many instances these deposits completely bridged the lumen, being attached to the artery wall at opposite sides and subdividing the original lumen into two smaller ones. Yet in no instance did such obstructing plugs incite the formation of thrombi. It was not unusual to find several calcified arteries in a single microscopic section. The larger arteries at the hilus of the lung were most often involved. No relation to the frequently coexistent bronchiectatic lesions could be demonstrated. Calcified arteries were found in 24.1 per cent of the animals whose pulmonary tissue was entirely normal. Arteries traversing the walls of large old abscesses were no more frequently calcified than those remotely situated.

Calcification of the pulmonary arteries was found in 224 or 46 per cent of the rats. The incidence would undoubtedly have been still greater if a more extensive microscopic examination had been carried out. 50.7 per cent of the males were involved and only 40 per cent of the females. The lesion was seldom found in animals less than 400 days of age at death and became progressively more prevalent until the 700th day of life, when its maximum incidence was attained.

The composition of the diets on which the animals were fed varied in calcium content, depending on the relative proportions of wheat and whole milk powder in the ingredients. Sherman and

Booher [10] have shown in this strain of rats that the total amount of body calcium in the growth period varied directly with the amount consumed. For this reason it was suspected that calcification of arteries might be influenced by the calcium content of the diet. When the animals were separated into two groups, one that had received diets containing about 0.19 per cent calcium, and the other with 0.33 per cent calcium, no difference in the incidence of arterial calcification between the two groups was noted. 55.4 per cent of those fed with the higher and 55.6 per cent of those with the lower calcium-containing diets had calcified pulmonary arteries. The reason why the percentage of calcification of both these groups is higher than that of the total series is that many of the animals dying at a young age were fed diets whose calcium content was not ascertained and are therefore excluded. Although the variations in calcium content cited above are not marked enough to exclude a possible dietary influence on the development of this lesion, it is obvious that in the present series its presence or absence did not depend upon the difference in calcium intake. It seems more likely that arterial calcification was a manifestation of local disturbance of calcium metabolism. The tendency to precipitate this mineral in extravascular situations as well, was quite prominent. Renal and vesical calculi were common and the bronchial cartilages were often calcified. The walls and contents of old abscesses were usually impregnated. The nature of this disordered calcium metabolism is not apparent. Although the parathyroid glands of senile rats often appeared enlarged and hyperplastic the bones showed no evidence of demineralization. The vitamin D content of the diet was not excessive.

Periarteritis: The arterial system of the rat is subject to a specific, often widespread inflammatory disease that has many of the attributes of periarteritis nodosa as it occurs in man. Lesions of this nature have been described in many different species. Nieberle [11] cited reports of its occurrence in cattle, swine, dogs and wild deer. Löwenthal [6] described several instances in which single arteries of old mice showed inflammatory changes of the same type. However no record of the lesion in the rat has been found.

In the present series 47 animals or 9.7 per cent exhibited evidence of the disease. Its preponderance in the female is noteworthy. 30 were females with an average age at death of 856 days

and 17 were males averaging 700 days in age. The incidence in different age groups was as follows: under 500 days, 0 per cent; from 500 to 700 days, 3 per cent; from 700 to 900 days, 13.1 per cent; and over 900 days, 15.7 per cent. Like so many of the other cardiovascular disorders in this species, the lesion was not found until late middle life and became more prevalent as age increased.

The lesion was apparently a long standing one and various stages in its development could be recognized. In 19 animals only acute or subacute lesions were found. In 6 there were only completely arrested and healed residua. In the remainder both healed and fresh lesions were intermingled. This latter finding was an indication that the disease may progress by a series of recrudescences. The extent and distribution of the process varied considerably although there were certain sites of predilection. When only 1 or 2 vessels were involved the process was classified as localized. When more than 2 arteries in different organs showed changes it was designated as generalized. 26 fell into the former category and 21 into the latter. Such a division is only approximately accurate since recognition of the lesion depended to a certain extent upon fortuitous microscopic sections. The changes were recognized grossly when the mesenteric arteries were extensively involved or when small aneurysmal outpouchings of arteries occurred elsewhere. The mesenteric arteries often showed striking alterations. The entire mesentery was enlarged and traversed by ropy, thick tortuous vessels which often appeared entangled with one another. The individual arterial branches were greatly enlarged. In fact they often exceeded the aorta itself in diameter. All along their course they were beaded by nodular protrusions which on closer examination were revealed as a series of aneurysmal dilatations. Many of these were occluded by thrombi. The earlier, more acute lesions were detected only on microscopic study. As a rule both large and medium sized arteries were attacked, the smaller arteries less often and the arterioles almost never. The aorta itself was spared. Lesions were at one time or another identified in almost every organ and tissue which were regularly examined save only in the lungs and brain. The frequency of involvement of various arteries was as follows: unidentified mediastinal and cervical, 21; mesenteric, 15; coronary, 15; pancreatic, 14; splenic, 11; renal, 11; gastric, 4. In addition the bronchial, hepatic, adrenal, uterine,

spermatic, ovarian, peripelvic and subcutaneous arteries showed the lesion on one or two occasions.

Microscopically there was much individual variation in the appearance of the lesions. A reconstruction of the pathogenesis of the process interpreted from all available material is as follows: The earliest change was the appearance of inflammatory cells in the adventitia often in eccentrically placed crescentic masses about the larger arteries and completely circumventing smaller ones. Multiple but discrete formation of such granulomas might appear along the course of a single vessel, producing a beaded effect. The cells consisted of mixtures of lymphocytes, plasma cells, monocytes, polymorphonuclear neutrophils and usually a few eosinophils. Often their nuclei became pyknotic and fragmented so that recognition of cell types was difficult. The cells lay between connective tissue fibrils, spreading them apart. Often the inflammatory changes encroached upon the outer aspects of the media, destroying smooth muscle cells. Before it penetrated the entire width of the media the endothelial lining might be elevated by a subintimal deposit of fibrin.

Still later the changes became continuous throughout the entire vessel wall, obliterating normal structures, destroying the media completely, disrupting elastic lamellas, and causing marked irregular thickening and narrowing of the lumen. The latter was often partly or entirely thrombosed. In some areas where the fixed tissue was most severely damaged aneurysmal widenings were common and there were sometimes hemorrhagic foci. Complete rupture was not seen. Usually by the time the process reached this stage considerable connective tissue had been formed in the adventitia providing new support. Healing was accomplished by a disintegration of the infiltrating leukocytes and replacement by dense scar tissue. The thrombi became organized and sometimes recanalized. The fibrous tissue assumed a hyaline appearance and on occasion calcium deposits were superimposed. There was some regeneration of smooth muscle but only fragments of the elastica persisted. All stages of development could be found in one animal and sometimes in a single artery. Areas of healing were not immune to secondary flareups and often an early fresh lesion was added to an older organizing one.

Save only in the case of the kidney, secondary manifestations

in the viscera due to obstructed blood supply were surprisingly infrequent. The gut was sometimes mottled by hemorrhagic areas. Focal necroses and interstitial scars appeared in the spleen, pancreas and myocardium. Definite infarction was not encountered. In the kidneys, however, there were often widespread tubular and glomerular changes indistinguishable in many respects from true glomerulonephritis. The convoluted tubules were widely dilated, lined by flattened epithelium, and obstructed by hyaline casts. Other areas of interstitial fibrosis and tubular atrophy although usually less frequent did distort the normal architecture. The glomeruli were in various stages of fibrosis and irregularly distributed in the cortex. Some were enlarged, others shrunken and completely hyalinized. Adhesions of tufts to the capsules of Bowman were numerous. The basement membranes of the capillary loops were thickened and the tufts themselves ischemic. Epithelial proliferation and cellular infiltration were never pronounced. Such changes in the kidney closely simulating if not identical with true glomerulonephritis were found in 9 of the 47 cases. In 13 others there were isolated areas of renal atrophy and fibrosis not unlike the scars resulting from arteriosclerosis of large renal arteries in man.

The etiology of this disease was not determined. Suppurative lesions were extremely common. In 18 of the 30 females there were large uterine abscesses. Similar foci of suppuration occurred with equal frequency in the absence of inflammatory arterial disease. Except for the involvement of the coronary arteries there was no association with endocardial or other intracardiac lesions. A possible relation to diet is disclosed by the fact that the disease appeared in only 1 animal out of 75 receiving meat and vegetables. In this animal the process was localized in the mesenteric artery. Among 356 rats known to lack either of these ingredients in their diet, there were 46 cases. The diet of these animals was less varied and consisted largely or entirely of dried milk powder and ground wheat. This discrepancy cannot be attributed to differences in longevity as 48 of the 75 animals in the first group survived longer than 700 days. Neither can it be ascribed to differences in susceptibility to suppurative infections since these occurred with approximately equal frequency and severity in both. It is thus suggested, although by no means proved, that dietary differences

may influence the incidence of this disease. It would require more extensive observations on a larger series of rats with the diets regulated from this point of view to establish the proposition.

There are many circumstances which make it seem probable that this disease is closely related to periarteritis nodosa in man. Certainly the histological features, the distribution of the lesions, the mode of onset and development, and the permanent residual deformities are closely analogous to those of the human disease. The pulmonary and cerebral arteries in both species are seldom involved. The mesenteric, renal and coronary arteries in both are often damaged. Again the vulnerability of medium sized arteries is a finding common to both. Renal lesions closely resembling glomerulonephritis have been described in association with the human form of periarteritis nodosa.[12] The occurrence of similar vascular lesions in many other species may indicate that they are all etiologically related. The only striking dissimilarities of rat to human periarteritis are in age distribution and incidence. The human disease occurs at all periods of life, whereas the rat lesion is pretty well limited to old animals. Human periarteritis is comparatively rare but in the rat it is one of the most common forms of systemic vascular disease. Neither of these discrepancies offers insurmountable evidence against the identity of the two processes.

Renal Lesions: The kidneys of senile rats are seldom entirely normal. In addition to the 9 cases of nephritis associated with periarteritis, there were 10 others in which the kidney cortex showed evidence of widespread degeneration involving both tubules and glomeruli. The only essential difference from human glomerulonephritis was the paucity of either proliferative or exudative reactions in the glomeruli and the fact that the kidneys were not contracted. Fine granulations of the cortical surface were sometimes visible with a hand lens. Another finding of high incidence was pelvic calculi leading to hydronephrotic atrophy. The kidneys were also subject to bacterial infection in many cases. Some of the older areas of scarring and atrophy were undoubtedly due to healed pyelonephritis.

Because of these complicating factors it was difficult to establish the extent to which the degenerative lesions encountered were dependent upon vascular disease. The major renal arteries seldom

deviated from normal although calcification was noted 8 times. The intima in senile rats was not thickened and the elastic fibers did not split or become multiple as is so often the case in adult man. Nevertheless there were many instances of linear or wedge shaped scars in the parenchyma directed at right angles to and retracted below the cortical surface. These had all the attributes of arteriosclerotic scars in the human kidney. In addition, a few scattered glomeruli were often hyalinized. Lesions of this nature were found in 65 males and 51 females, a total incidence of 23.8 per cent. They were uncommon in animals younger than 700 days at death and present in 34.6 per cent of those surviving more than 900 days. Because of the absence of sclerotic changes in the renal arteries it is impossible on morphological grounds to associate the scarring with impaired circulation. Some must have resulted from healed pyelonephritis, others possibly from pressure on the arteries in the peripelvic tissue exerted by intrapelvic calculi. This latter suggestion is not entirely implausible inasmuch as the peripelvic tissue in the rat is lacking in adipose tissue which might cushion and protect the vessels. The arterioles in and about the scars often seemed to have thick muscular walls and only minute lumens, but hyaline necrosis was not observed.

The Arterioles

In general, the arterioles showed little pathological change. In no instance were arteriolarsclerotic lesions disseminated throughout the splanchnic area, as is seen so frequently in association with human hypertension. There is no reason for believing that any of the rats had had elevated blood pressures comparable to a primary hypertension. Hypertrophy of the heart occurred only in conjunction with other cardiac lesions or in animals that had severe renal lesions. As previously noted, in scarred kidneys the arterioles sometimes appeared thickened and tortuous. Definite hyalinization of the renal arterioles was observed in 3 cases and in only 1 of these were the changes striking. In this particular animal, a 1005 day old female, there were associated widespread glomerular changes which might have been secondary to the arteriolar lesion. The renal changes were suggestive of arteriolar nephrosclerosis but extrarenal arteriolar lesions were not observed.

The splenic arterioles were examined closely inasmuch as these

are so constantly altered in man. In only 1 animal, an 800 day old female, were comparable lesions found. In the spleen of this particular animal practically every follicular arteriole was thickened and contained a subintimal accumulation of deeply eosinophilic, homogeneous material. In the spleen of 1 other rat, an 850 day old male, a few central arterioles showed similar changes. In all the other old animals the splenic arterioles were perhaps thicker and more tortuous than normal but there were no definite sclerotic changes.

The pulmonary and bronchial arterioles often exhibited striking muscular hypertrophy. This, however, was usually associated with more profound changes in the larger arteries.

The Veins

The only finding of note in the veins was the occasional thrombosis of the hepatic, adrenal, splenic, renal, uterine and pulmonary vessels. The thrombi were at times secondary to lesions in the adjacent tissue but also formed in normal tissue without obvious cause. Small pulmonary emboli were present in a few cases and were probably derived from such thrombi. With advancing age none of the veins examined showed degenerative changes of any consequence.

Summary and Conclusions

The pathological manifestations of vascular disease in 487 rats of all ages in which death occurred as the result of natural causes are described. Intimal lesions of the arteries comparable to those of man and birds, or those experimentally induced in rabbits following cholesterol ingestion, or in the coronary arteries of rats with administration of excessive doses of vitamin D (Ham and Lewis [13]), were not observed. The elastic fibers in the aortas of senile rats were thicker and less undulating than those of young ones. Except for the absence of fraying and splitting, this change is analogous to that in the elastic fibers of man with advancing age. If it be interpreted as an indication of reduced elasticity, the assumption can be made that degeneration of elastic tissue is dependent upon the natural life span and not upon simple aging of this tissue. Since intimal thickening did not accompany this medial change, as it so commonly does in man, it seems likely that the two

are unrelated and that the latter is not a true phenomenon of senility.

Only the coronary and pulmonary arteries were commonly the seat of degenerative changes. These consisted of fibrosis of the media and thickening of the wall. In the pulmonary arteries the lesion was frequently associated with calcification. Calcification was found also in other arteries, particularly the spermatic.

A specific inflammatory disease of arteries identical with or at least closely resembling periarteritis nodosa in man was found in 9.7 per cent of the animals.

Renal lesions similar to arteriosclerotic atrophy in the human kidney were described but their association with vascular disease could not be established. The arterioles showed evidences of sclerotic changes in only a few exceptional cases and in none was generalized arteriolarsclerosis recognized.

All of the lesions encountered were influenced by age, few of them being observed before the 700th day of life. All of the non-inflammatory lesions were more common in males.

The absence of amyloidosis in all of the animals is particularly noteworthy in view of the success with which this change has been experimentally produced in rodents by a variety of methods.[14] The circumstances would seem to have been especially propitious for its development spontaneously in these animals. Chronic suppurative lesions were very common and one of these, infection of the auditory bullae, was often associated with osteomyelitis of the adjacent bony structures sometimes with extension into the cranial cavity.

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DESCRIPTION OF PLATES

PLATE 40

FIGS. 1 and 2. Elastica in media of aorta. The photographs are from comparable points in the ascending aorta. Fig. 1 is from a 93 day old and Fig. 2 from a 1021 day old rat. In the senile animal the media is much broader, the individual lamellae slightly thickened and much farther apart. The most striking change, however, is the stretching and loss of undulation of the elastic fibers in Fig. 2, as compared to those in Fig. 1. Weigert's elastic tissue stain. × 300.

FIG. 3. Calcification of pulmonary artery. Two solid masses of calcium are embedded in the intimal surface of the sclerotic vessel. The wall of the artery at this point is irregularly thickened by dense fibrous tissue which has replaced the smooth muscle. × 300.

FIG. 4. Calcification of abdominal aorta. At the orifice of a large arterial branch a solid mass of calcium is deposited and protrudes into the lumen. × 100.

1 2

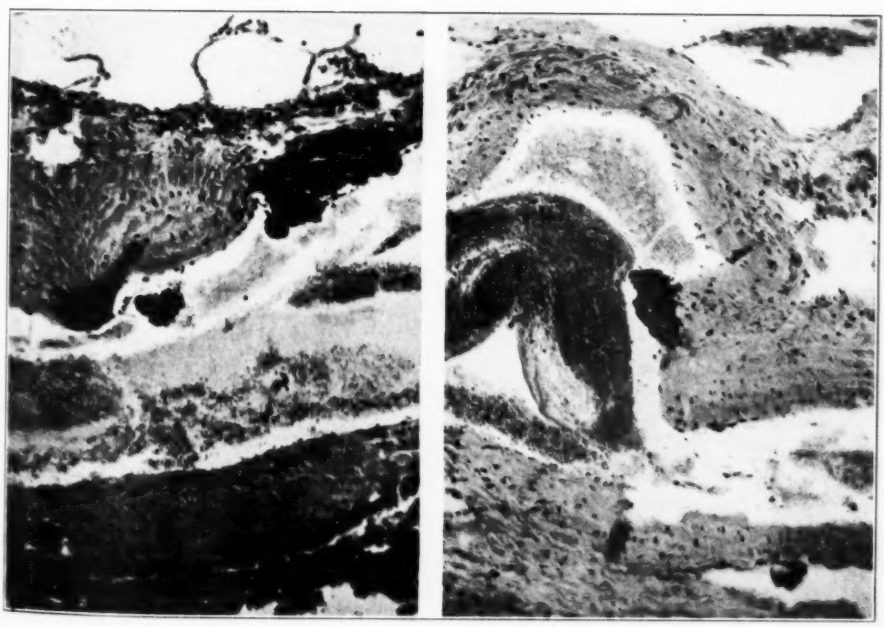

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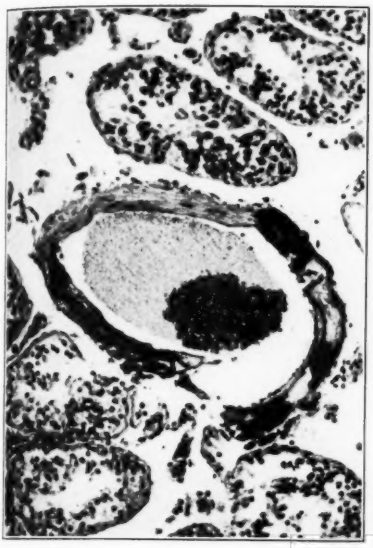
PLATE 41

FIG. 5. Calcification of spermatic artery. Calcium is deposited as curved plates in the media of the vessel without causing the latter to become thickened. × 110.

FIG. 6. Acute periarteritis of small artery. The entire adventitia of the vessel is heavily infiltrated by lymphocytes, polymorphonuclear leukocytes, large mononuclear cells and pyknotic nuclei. The media is degenerating and a subintimal deposit of fibrin has been precipitated. The lumen is still patent. × 460.

FIG. 7. Acute periarteritis of large peripancreatic artery. An acute inflammatory reaction attended by the fragmentation of nuclei of infiltrating cells is apparent in the adventitia and outer aspects of the media. The nodular character of the lesion is self evident. × 110.

FIG. 8. Chronic periarteritis of mesenteric arteries. The arteries throughout the mesentery to their points of entrance into the intestinal wall are greatly enlarged, twisted, tortuous and nodular. Aneurysmal dilatations are numerous.



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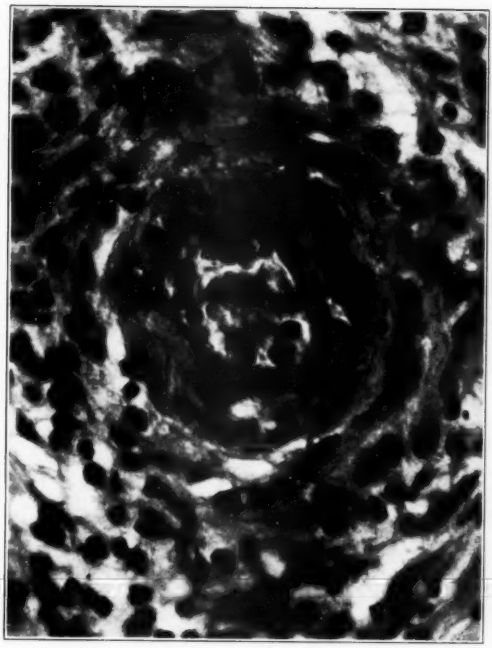

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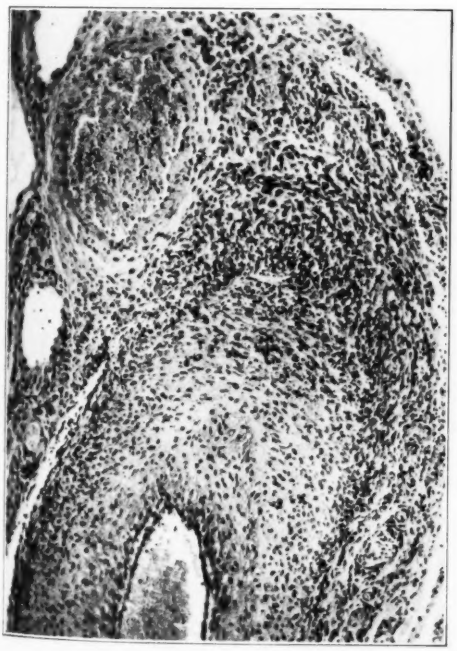

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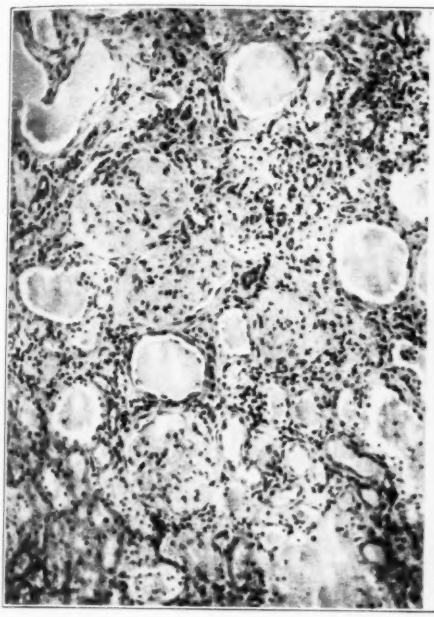
PLATE 42

FIG. 9. Nephritis in periarteritis nodosa. The architecture of the cortex is disarranged. Some of the tubules are enlarged and obstructed by hyaline casts. Others are atrophic and have shrunken into the increased interstitial connective tissue. The latter is infiltrated by lymphocytes. The glomeruli are distorted and swollen so that the tufts obliterate the capsular spaces. The tufts are ischemic, compact and depleted of cells. × 110.

FIG. 10. Atrophy and fibrosis of renal cortex. The edge of a wedge shaped scar borders on adjacent intact renal cortex and merges with the slightly thickened and sunken capsule. The tubules in the scar are completely atrophic and the glomeruli are shrunken and partly replaced by fibrous tissue. A heavy lymphocytic reaction has occurred. × 110.

FIG. 11. Hyalinization of splenic arterioles. The lumens are greatly narrowed. The walls are thickened by dense homogeneous masses of hyaline material lying between the endothelium and the outer layers of the vessel wall. × 110.

FIG. 12. Marked smooth muscle hypertrophy in the media of a pulmonary arteriole. The lumen is greatly reduced. A small capillary is seen as it emerges directly from the arteriole. × 300.



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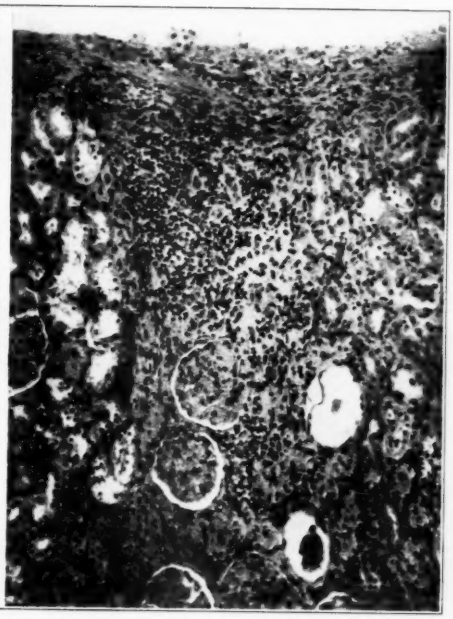

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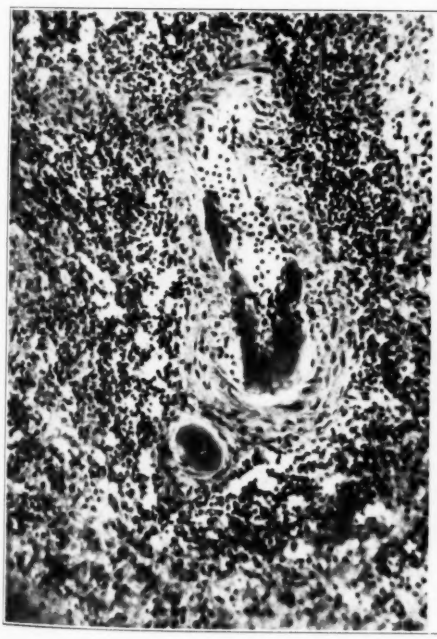

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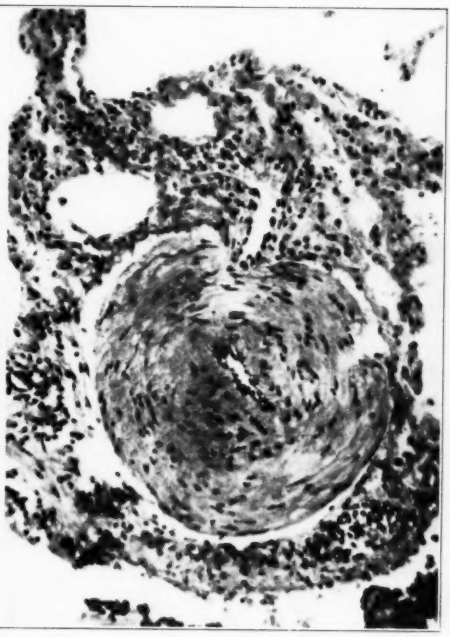

12

TRANSMISSION OF CHLOROLEUKEMIA OF MICE *

JOHN W. HALL, M.D., AND FREDERICK J. KNOCKE, B.S.

(From the Department of Pathology, Cornell University Medical College, New York, N. Y.)

Since Burns described the first case of chloroleukemia in man in 1825, 175 cases of this disease have been reported up to 1937.[1] Early reports considered chloroleukemia to be of the lymphoid type, but recent cases have all been classified as myeloid in type.[2, 3]

Chloroleukemia differs from other leukemias only in the green color of the leukemic nodules and lymph nodes. The shade of green varies in different cases and even in the different tissues of the same case. The green color has been attributed to the presence of lipochromes,[4] porphyrines,[5] and the eosinophils,[6] which abound in some cases of chloroleukemia. Treadgold [7] believes that the green color is possibly due to a degeneration of the granules of the myelocytes and myeloblasts, aided by the products of hemoglobin disintegration. It has been suggested by Kossel and Giese [8] that the green color depends on the presence of both free sulphur ions and a certain amount of iron in a reactive state.

Chloroleukemia has been reported in several of the domestic and laboratory animals, including the pig, common fowl, rat and mouse. The term chloroleukemia in fowl, as used by Mathews,[9] is a misnomer, since the multiple tumors of the fowl diagnosed by him as chloroleukemia have a white color and are not known to assume a greenish hue.

Wilens and Sproul [10] reported 12 cases of spontaneous leukemia in the rat, 1 of lymphoid and 11 of the myeloid type. In 4 of the myeloid cases the leukemic tissues were light green in color.

Simonds,[11] describing the 67 cases of leukemia in the first 15,000 autopsies of the Maud Slye strain of mice, found no cases of chloroleukemia. The only case of this disease in mice was reported by Hill,[12] in an article describing lymphoid hyperplasia in 215 mice. She describes one mouse with "chloro-myelo-sarcoma," in which there was a mixed myeloid and lymphoid invasion of the

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lymph nodes and spleen. The blood picture, however, was myeloid in nature. No attempt was made to transmit the disease.

The transfer of chloroleukemia and the anatomical characteristics of this disease in mice are the subject of this paper.

A case of chloroleukemia in a mouse was observed in this laboratory in November, 1936. Hundreds of cases of spontaneous lymphoid leukemia, and numerous cases of myeloid leukemia, have occurred here, but the green color of the leukemic infiltrations has been observed only in very few mice. This mouse, Slb 351, was a female, born Nov. 3, 1935, and dying Nov. 8, 1936. At postmortem the spleen was grayish red and enlarged to 3.5 by 1.2 cm. The superficial lymph nodes were enlarged to 4 to 7 mm. in the greatest diameter and were a light green in color. The internal lymph nodes were similar to the superficial lymph nodes, both in color and in size, but the mediastinal nodes were not involved. The liver was slightly enlarged and gray. In the lungs there were numerous small red areas of hemorrhage. A blood smear showed numerous erythroblasts and the white cells were estimated at 400,000 with myeloid cells in all stages of maturity, myeloblasts being especially numerous.

Material and Methods

The spleen and lymph nodes were removed aseptically, minced in the presence of Tyrode solution, and drawn into a syringe through cotton to filter out the larger particles. Intravenous injections of 0.1 cc. of this suspension were made into the tail vein. Subcutaneous injections of 0.2 cc. of unfiltered fragments of leukemic tissue were made into the subcutaneous tissue of the right side. Later, with the development of subcutaneous tumors, tumor material was prepared as described above and injected intravenously and subcutaneously.

Transfers were also made of splenic material which had been frozen slowly to −70° C. during one-half hour and kept at that temperature for periods ranging from 6 to 93 days. (Concerning the preservation of leukemic cells in the frozen state, see Breedis, Barnes, and Furth.[13]) The material was thawed slowly by keeping the sealed tubes containing the frozen splenic material first at refrigerator and subsequently at room temperature. A cell suspension was made of the splenic material and injected intravenously.

Differential cell counts were made on touch impressions of the leukemic organs and tumors and on blood smears stained with Wright's and Giemsa's solutions and with the benzidine oxydase stain.

Experimental

Approximately 2 hours after the death of mouse Slb 351, during which time it was kept in the refrigerator, the spleen and lymph nodes were separately cut up in Tyrode solution. Of 5 mice injected intravenously with splenic suspension, 3 developed chloroleukemia; while 2 mice injected intravenously with a cell suspension of lymph node developed chloroleukemia.

Table I

Susceptibility of Related Mice to Various Routes of Injections of Spleen, Lymph Nodes and Leukemic Tumor Tissue

Route of injection	Number of mice injected	Successful inoculations
Intravenous	82	78
Subcutaneous	13	3
Intravenous and subcutaneous	10	8

During a period of 12 months numerous transfers were made. Table I summarizes the results obtained by the injection of spleen alone, spleen and tumor, and spleen and lymph node by intravenous, subcutaneous and combined subcutaneous and intravenous injections. Of the different routes, intravenous was the best, being successful in 95.1 per cent of the injections. Subcutaneous tumors were observed following subcutaneous inoculation in 23 per cent of the mice inoculated.

The length of life following intravenous injection of splenic material averaged 21.5 days in a series of 31 mice. The longest duration of life following injection was 48 days, and the shortest 9 days. A tendency towards a decrease of length of life following repeated passages was observed. For the first 6 passages the average length of life was 26.7 days, whereas in the last 6 passages the duration of life was 16.6 days.

Subcutaneous tumors could be as readily produced by the use of

tumor tissue alone as with spleen or lymph nodes. The production of generalized chloroleukemia was approximately one-third as frequent in the mice injected intravenously with tumor cell suspension (30 per cent) as in those mice injected intravenously with cell suspensions of other tissue, spleen and lymph nodes (95.1 per cent) (Table II).

Of five mice that were given a single dose of X-ray (400 r), all developed subcutaneous tumors 14 days following the subcutaneous inoculation of a suspension of splenic tissue (Table II). While there was a rapid development of subcutaneous tumors at the site of inoculation, these mice did not show evidence of generalized leukemia until from 55 to 75 days after subcutaneous inoculation.

TABLE II

Results Obtained by the Injection of Tumor Material into Related Mice

Route of injection	Number of mice injected	Successful inoculations
Intravenous	10	3
Subcutaneous	23	5
Subcutaneous *	5	5

* Mice given 400 r preceding the inoculation.

Twenty mice of unrelated stock were injected both subcutaneously and intravenously with a cell suspension of splenic tissue. Half of these mice were irradiated (400 r). None of the mice developed leukemia. Eight mice were then injected intravenously with a cell suspension of spleen after exposure of the mice to 400 r of X-ray and 1 week later these mice were given 300 r of X-ray. At the end of 10 weeks none of these mice had developed leukemia.

Reinjections were made into mice that had failed to develop leukemia after they had been injected intravenously or subcutaneously with leukemic cells. The reinjections were made intravenously, but none of the 14 mice developed leukemia. These mice either had a natural resistance to leukemia or had been immunized by the original inoculation.

Cell suspensions were made of the splenic material which had been frozen at −70° C. and kept at that temperature for varying periods of time. Leukemia developed in 1 of 4 mice injected with

splenic material kept at −70° C. for 6 days. Of 4 mice injected with splenic material kept at −70° C. for 13 days, 1 developed leukemia; in 5 mice injected with the same material frozen at −70° C. for 94 days, leukemia failed to develop.

Of all strains of leukemia, this was found to be the most susceptible to freezing; nevertheless, a sufficient number of cells survived to produce leukemia in 2 of 13 mice injected. Further experiments on the susceptibility of these cells to freezing are in progress.

Gross and Microscopic Examination of Tissues of Mice with Generalized Chloroleukemia and Subcutaneous Tumors

The mouse that developed the spontaneous chloroleukemia and the intravenously injected animals developed the same type of diffuse, generalized lesions, but no tumor formation was observed. The following description is characteristic of most of the mice that developed the generalized disease.

The spleen is greatly enlarged, measuring up to 3 by 1.3 by 0.8 cm., and is grayish red in color. There is generalized enlargement of the lymph nodes. The cervical, axillary, inguinal, mediastinal and periaortic nodes measure from 3 to 8 mm. in the greatest diameter and are of various shades of green. The mesenteric nodes measure 2 by 0.6 by 0.3 cm. and are similar in color to the other lymph nodes. The bone marrow is grayish red. The liver is moderately enlarged. It is light reddish brown mottled with small irregular areas of red and grayish yellow. The lungs, both externally and on the cut surface, show small irregular areas of red. In some instances the kidneys are pale red and contain minute areas of gray. The heart, adrenals, intestine and ovaries appear normal in gross.

Microscopically, the splenic pulp is extensively infiltrated by leukemic cells. These cells for the most part are of two types: (1) large cells with large, irregular, rounded, either hyperchromatic or vesicular nuclei, and very little cytoplasm; the vesicular nuclei show large acidophilic nucleoli; and (2) large cells with bean shaped nuclei in which there is a greater amount of cytoplasm than in the above mentioned cells. Mitotic figures are numerous. Very few immature leukocytes are present. The lymph follicles

are atrophic or have been replaced by leukemic cells. A moderate number of megakaryocytes are present. In the other organs and structures in which there are leukemic infiltrations, the same types of cells are present, with the exception that megakaryocytes are not present. The normal structure of the lymph node is replaced by an infiltration of leukemic cells. Small areas of beginning necrosis are present. The capsule and pericapsular tissue are also infiltrated with leukemic cells. In the bone marrow there is replacement of the normal structure by masses of myelocytes and myeloblasts. The periosteum is raised from the cortex of the bone by a thin layer of leukemic cells. This layer is in direct continuity with the leukemic cells in the marrow cavity by way of growths of leukemic cells in the central canals of the Haversian systems (Fig. 7). In the liver there is extensive infiltration of the portal spaces by leukemic cells. Small collections of leukemic cells are also present in the liver lobules away from the portal spaces. A moderate number of mitotic figures are seen in these groups of cells. There are many leukemic cells in the liver sinusoids, which contain more leukocytes than erythrocytes (Fig. 4). A few erythroblasts are also present in the sinusoids. In the kidneys there are extensive infiltrations of leukemic cells around the blood vessels in both the cortex and the medulla. The glomerular capillaries are filled with leukemic cells. The alveolar walls of the lungs are greatly thickened by infiltrations of leukemic cells, to such an extent that in some areas the alveoli are collapsed and solid fields of leukemic cells are present. Conspicuous infiltrations are seen at the hilum of the lung around the vessels and bronchi (Fig. 3).

The subcutaneous leukemic tumors present at the site of injection vary in size from 0.6 by 0.4 by 0.4 cm. to 1 by 1 by 0.8 cm. The tumors range from a grayish yellow to a light green in color, and are attached to the subcutaneous tissue and in some cases to the overlying skin.

Microscopically the leukemic cells have infiltrated the subcutaneous tissue and skeletal muscle. Many of the cells are large with very little basophilic cytoplasm. The nuclei of some are hyperchromatic, while most are vesicular with acidophilic nucleoli. There are also a few cells with bean shaped nuclei and with a large amount of cytoplasm. A very few immature leukocytes are present.

Eosinophilic leukocytes are not present in the tumor but are seen occasionally in the spleen. Mitotic figures are extremely numerous (Figs. 5 and 6).

Differential counts made with Wright's and Giemsa's stains and with oxydase stained blood smears indicate that approximately 50 per cent of the cells are myeloblasts, promyelocytes, myelocytes and metamyelocytes. There are a moderate number of annular forms and immature polymorphonuclear leukocytes present, but mature polymorphonuclear leukocytes are never more than 5 per cent (Figs. 1 and 2). In the bone marrow and leukemic tissues, studied by means of Wright's and Giemsa's methods, and oxydase stained touch impressions, 75 to 85 per cent of the cells are myeloblasts, promyelocytes, myelocytes and metamyelocytes. The subcutaneous tumors are composed of from 80 to 95 per cent myeloblasts and promyelocytes with no evidence of maturation towards myelocytes and metamyelocytes.

White blood counts done on 5 leukemic mice ranged between 136,900 and 672,000 per cmm.

Observations on Green Color of Lymph Nodes

The green color of the lymph nodes fades rapidly following exposure to air, and has entirely disappeared in 15 minutes to a half hour. When the material is preserved in Kaiserling's solution the color fades, as when exposed to the air.

An attempt was made to preserve the color, using a reducing solution of sodium hydrosulphite,[14] without success. Hydrogen peroxide has often been recommended for the preservation of the green color of chloroleukemia.[15] Several attempts were made to preserve the green color by the use of 3 per cent hydrogen peroxide but the green color faded more rapidly in the solution than in air.

Microspectroscopic examination of the green lymph nodes did not reveal any characteristic band.

Microscopic examination of the green colored tissues indicated that the green color was not due to the presence of eosinophils, as these cells were not present at all, or only in very small numbers.

Placing the green tissue in Tyrode solution in an atmosphere of carbon dioxide preserved the color very satisfactorily for from 3 to 4 hours.

Summary and Conclusions

A strain of chloroleukemia of mice is described that is readily transmitted to related mice by the intravenous injection of a suspension of leukemic cells.

Subcutaneous inoculation of leukemic leukocytes produces a localized tumor at the site of inoculation in approximately 23 per cent of the inoculated mice. These tumors grow very slowly. Intravenous injection of a suspension of leukemic cells produces a rapidly progressing generalized leukemia, fatal after approximately 20 days, in 95.1 per cent of the injected mice. This observation indicates that large numbers of leukemic cells are destroyed in the subcutaneous tissues of mice that are susceptible to intravenous administration of similar cells.

Suspensions of tumor cells injected intravenously are much less effective in transmitting the disease than spleen and lymph node.

Tumor tissue and splenic tissue subcutaneously injected are about equally effective in producing subcutaneous tumor nodules.

Exposure of mice to 400 r of X-ray preceding the injection results in a greater percentage of successful subcutaneous inoculations.

Unrelated mice of two different stocks are resistant to transmission of the disease. Exposure of these mice to 400 r of X-ray has not rendered them susceptible to the disease.

Mice that have been negative following intravenous or subcutaneous injection of leukemic cells have also been negative following intravenous reinjection with leukemic splenic material.

The almost complete absence of eosinophils in the leukemic infiltrations indicates that these cells are not responsible for the green color. The most intense green color is shown by the lymph nodes, while the subcutaneous tumors, which are composed almost exclusively of malignant leukemic cells, are gray with only a faint greenish hue.

Note: We wish to express our thanks to Dr. Jacob Furth for his many helpful suggestions and advice which aided us in the completion of this study.

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DESCRIPTION OF PLATES

All blood smears were stained with Wright's and Giemsa's solutions and the sections with hematoxylin and eosin solutions. The magnifications stated are approximate.

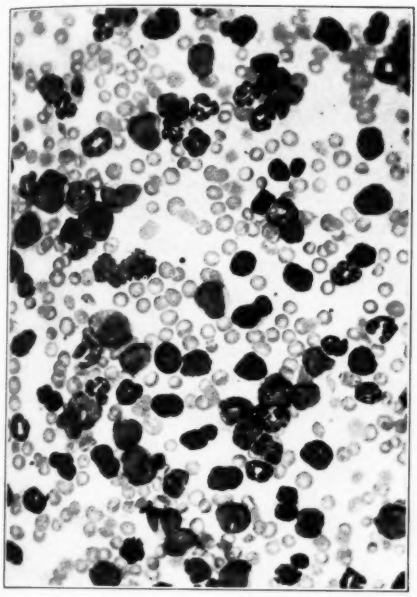
PLATE 43

FIG. 1. Blood smear from a mouse with transmitted chloroleukemia, showing myeloblasts, promyelocytes, myelocytes, premyelocytes and immature polymorphonuclear leukocytes. × 300.

FIG. 2. Higher magnification of a blood smear from a mouse with transmitted chloroleukemia, showing immature myeloid cells. × 900.

FIG. 3. Extensive myeloid infiltration of lung around a bronchiole and a blood vessel in a mouse with transmitted chloroleukemia. × 100.

FIG. 4. The liver from a mouse with transmitted chloroleukemia, showing distention of the sinusoids by leukemic cells, with compression of the liver cells and extensive infiltrations in the portal spaces. × 200.



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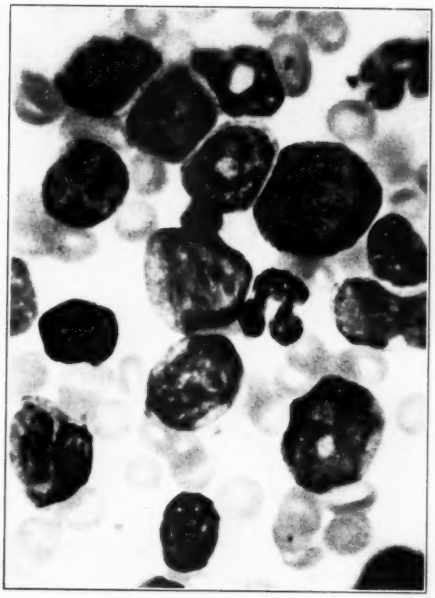

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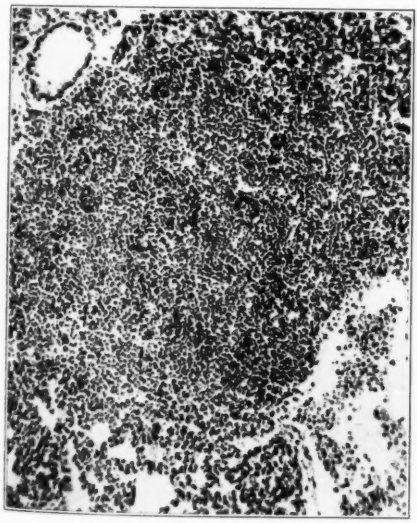

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4

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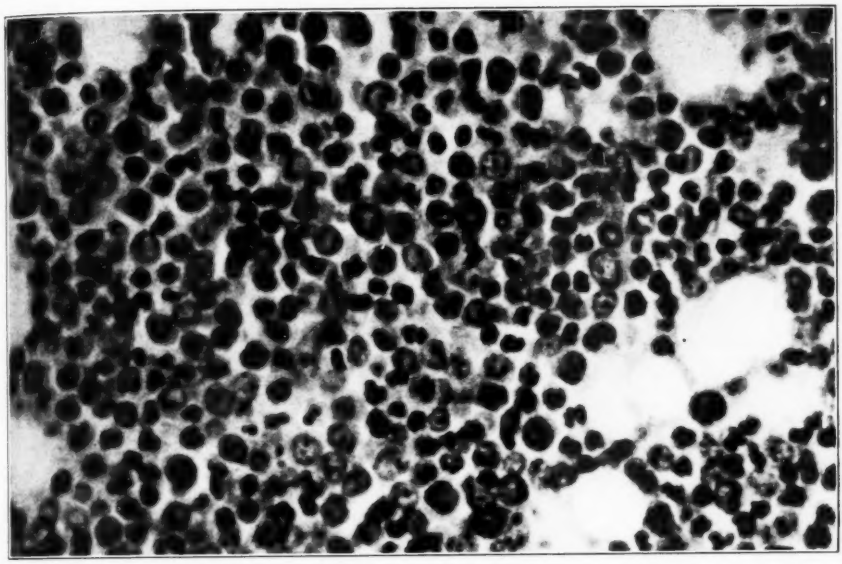
PLATE 44

FIG. 5. Immature myeloid cells infiltrating the subcutaneous tissue following subcutaneous inoculation. × 700.

FIG. 6. Higher magnification of subcutaneous tumor showing myeloblasts, promyelocytes and several more mature myeloid cells. Several mitotic figures are present. × 900.

FIG. 7. Advanced infiltration of the bone marrow by leukemic cells with extension through the Haversian canals and separation of the periosteum from the cortex. × 200.



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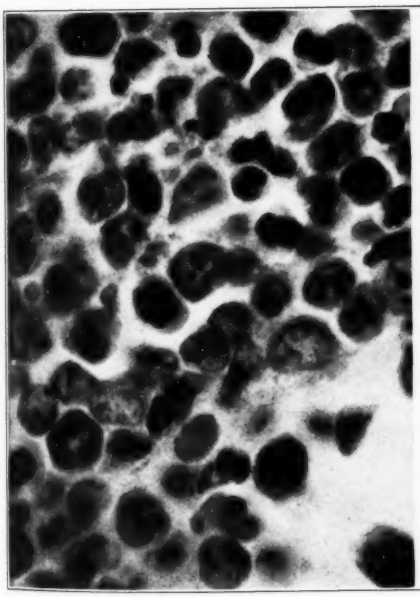

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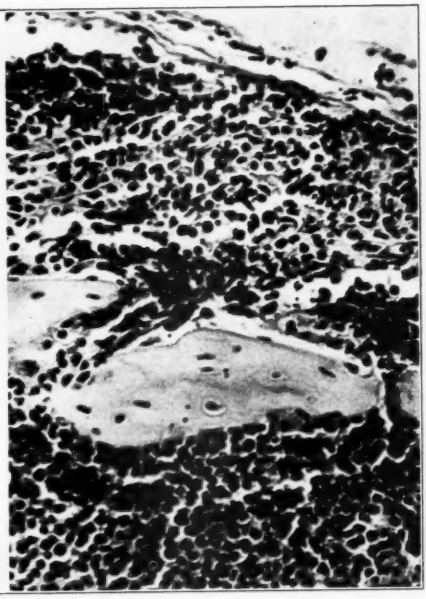

7

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Transmission of Chloroleukemia of Mice

THE DISTRIBUTION OF MATERIAL FOLLOWING INTRACEREBRAL INOCULATION INTO MACACUS RHESUS MONKEYS AND ITS POSSIBLE INFLUENCE UPON THE RESULTS OF NEUTRALIZATION TESTS IN EXPERIMENTAL POLIOMYELITIS *

MORRIS SCHAEFFER, PH.D., AND RALPH S. MUCKENFUSS, M.D.
(From the Bureau of Laboratories, Department of Health, New York, N. Y.)

Intracerebral inoculations of monkeys with poliomyelitis virus have been employed extensively since Flexner and Lewis [1] in 1909 demonstrated that this route of inoculation is an effective method of transmitting the disease to these animals. Because monkeys have been most consistently infected with virus in this manner, the intracerebral inoculation has been considered the most reliable means of determining infectivity of the virus, especially after it has been subjected to treatment with an inhibitory reagent such as immune serum. However, despite the widespread use of injections into the monkey brain, there is little knowledge concerning the fate of the inoculum subsequent to its deposition into the brain substance.

Many investigators [2-5] have studied the diffusion of material throughout the central nervous system by the injection of dyes or of India ink; but as far as we could ascertain, no experiments have been conducted with a view toward determining to what degree and extent material introduced into an area of the brain is eventually distributed, and what bearing the resultant distribution may have upon the ultimate infectivity of infectious material thus deposited.

Hurst,[6] in a study on the pathogenesis of experimental poliomyelitis, as a preliminary step injected India ink into the cisterna magna of a monkey in order to follow the course of diffusion. Two days later the ink had penetrated into the meninges along the whole length of the cord, over large areas of the brain stem, cerebellum, base of the cerebrum, along certain of the fissures, about half way up the lateral surfaces of the hemispheres, the choroid plexuses and the lateral ventricles. The nervous substance itself was not

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discolored, except for slight staining in the floor of the fourth ventricle.

When virus was inoculated intrathecally the earliest lesions were usually situated in the floor of the fourth ventricle. This suggested to Hurst that the penetration of the virus through the nervous tissue may occur at the ependyma of the fourth ventricle where, under experimental conditions, the virus is regurgitated at operation; but the cerebrospinal fluid does not necessarily participate in its spread through the nervous system.

Experimental

The work to be presented here is an outgrowth of a series of experiments conducted on the neutralizing action of immune serum upon the virus of poliomyelitis.* During the course of this study numerous discrepancies in the results were observed, and an effort was made to determine what possible factors might be responsible for such variations. Among other things, the inoculation procedure and its effect upon the results were investigated.

Experiment I

To follow the course of distribution of substances from the site of inoculation, material containing India ink was introduced into the brains of *Macacus rhesus* monkeys in the manner usually employed in our previous experiments.

Technique: The customary serum-virus mixture used in our neutralization tests was prepared. This consisted of 1.5 cc. of a Berkefeld N filtered 5 per cent suspension of poliomyelitic monkey spinal cords, mixed with 1.5 cc. of human convalescent serum. To this mixture, 1 cc. of India ink was added. After thorough mixing, the material was injected into the right frontal lobes of 4 monkeys, each receiving 0.25 cc., 0.5 cc., 1 cc., and 2 cc., respectively, with a tuberculin syringe carrying a ¾ inch, 26 gauge needle, which was inserted through a trephined opening made in the frontal bone approximately 1 cm. to the lateral right of the midline and 1 cm. anterior to the coronal suture. After 2 hours the animals were chloroformed and their brains and cords examined at autopsy. The results of this experiment are summarized in Table I.

* The details of these experiments and a review of this subject, which entail some length, will be published elsewhere.

Experiment II

A second series of 4 monkeys was inoculated in a manner similar to those in Experiment I. In this group 2 monkeys received 1 cc. and 0.25 cc., respectively, of the serum-virus-ink mixture with a ¾ inch, 26 gauge needle, and 2 others were injected with 1 cc. and

TABLE I

The Diffusion of Material Inoculated Intracerebrally (Right Frontal Lobe) Throughout the Central Nervous System, as Evidenced by the Distribution of India Ink Contained in the Inoculum

Monkey No.	Amount of mixture injected	Appearance of the central nervous system 2 hours after injection
	cc.	
1	0.25	There was evidence of seepage of the material from the brain substance into the subarachnoid space above the site of inoculation. Slight hemorrhage at the site of inoculation was also noted. No India ink was observed on the spinal cord (Figs. 1 & 2)
2	0.5	When the monkey was chloroformed, leakage was observed to be still taking place externally at the site of injection. On flapping back the scalp India ink was found to be deposited around the trephined opening and the surrounding area (Fig. 3). The material had diffused over the surfaces of the brain, cerebellum and spinal cord (Figs. 4 & 5). Sections at the site of inoculation showed that the injection had probably been made directly into the lateral ventricle (Figs. 5 & 6)
3	1.0	This monkey had been used in a neutralization test 2 months previously and therefore, as is often observed, had a sterile abscess in the right frontal lobe at the previous site of inoculation. On examination it was noted that although some India ink had seeped into the subarachnoid space, the bulk of the inoculum was found to be confined to the necrotic cavity on the right side (Fig. 7)
4	2.0	The entire surface of the brain and cord of this animal was covered with India ink, indicating the extensive seepage of the inoculum from the site of inoculation into the cerebrospinal fluid (Figs. 8 & 9)

0.25 cc., respectively, using a ½ inch, 26 gauge needle. After 2 hours the animals were sacrificed and their brains and cords examined at autopsy. The findings are summarized in Table II.

From the results of these experiments it was evident that the distribution of material following intracerebral deposition varied

to some extent. In most cases, however, little of the material remained at the site of inoculation but rapidly entered the cerebrospinal fluid either by seeping backward through the path of inoculation into the subarachnoid space or via the ventricles into the spinal canal. Except for areas reached by the needle no carbon

TABLE II

Comparison of the Diffusion of Material Through the Central Nervous System after Intracerebral Inoculation with Needles of Two Sizes

Monkey No.	Amount of mixture injected	Size of needle	Appearance of the central nervous system 2 hours after injection
	cc.		
1	1.0	½ inch	The entire surface of the right frontal lobe (the side inoculated) up to the fissure centralis was covered with India ink (Fig. 10). No ink was observed on the opposite hemisphere or on the spinal cord. Sections examined at the site of inoculation indicated that the inoculum had been deposited in the brain substance at the site of inoculation below the cortex (Fig. 11), but some seeped backward and entered the subarachnoid space
2	0.25	½ inch	The general appearance of the brain and cord of this animal was similar to the one above, except that there was less distention of the brain tissue at the site of inoculation (Fig. 12)
3	1.0	¾ inch	The entire cerebral hemisphere opposite to the side inoculated was completely covered with India ink (Fig. 13). Ink was also present in all of the ventricles, the base of the brain and the spinal cord (Fig. 14). The photograph clearly shows the path of the needle
4	0.25	¾ inch	No ink was observed on the surface of the brain, but it was abundant on the surface of the spinal cord (Fig. 15). A section of the brain revealed a considerable quantity of ink in the lateral ventricle (Fig. 16)

particles were found deposited in the nervous substance itself, but the India ink adhered to the surfaces of the brain or cord. There was some suggestion that with a shorter needle and a smaller amount of material the inoculum did not as readily diffuse through the cerebrospinal fluid and reach the spinal cord. On that basis,

therefore, neutralization tests were performed to compare the results of inoculation of a large and small volume of material, using needles of ⅞ inch and ¼ inch length. The ⅞ inch needle was employed in order that the inoculum might reach the lateral ventricle, and the ¼ inch needle in order that the material might be deposited into the cerebral cortex. The volumes selected were 1 cc., the usual amount inoculated in our neutralization tests, and 0.25 cc., the injection of which in previous experiments had indicated a tendency toward greater regularity.

Experiment III

Technique: A set of 10 duplicate test tubes, each containing 1.5 cc. of a 5 per cent virus filtrate and 1.5 cc. of pooled human convalescent serum, was incubated for 2 hours at 37° C. and kept in the refrigerator overnight. From each of 5 of these tubes 2 monkeys were inoculated intracerebrally with 1 cc. and 0.25 cc., respectively, using a ⅞ inch needle. From each of the remaining 5 tubes 2 monkeys respectively received 1 cc. and 0.25 cc. with a ¼ inch needle. Immediately before injection 0.25 cc. of sterile India ink was added to each tube in order that the dispersion of the inoculum could be followed in those animals that developed poliomyelitis. The results are summarized in Table III.

The variable manner in which material inoculated intracerebrally diffuses is again illustrated by this experiment. The extent of the distribution is apparently not entirely governed by the amount inoculated or the length of the needle employed. It is interesting to note, however, that none of the monkeys receiving material with the ¼ inch needle developed poliomyelitis, while 4 of the 10 monkeys inoculated with similar mixtures, but with ⅞ inch needles, became infected.

It has been observed [7-10] that upon dilution of a neutral mixture of virus and immune serum a subsequent disruption of the virus-serum union, the so-called dilution phenomenon, takes place and the mixture again becomes infective. The results of the above experiments suggested the possibility that if some quantity of the inoculum escapes from the area of inoculation into the cerebrospinal fluid, the dilution phenomenon may occur within the animal body and thus account for the occasional infectivity of an otherwise apparently inactivated mixture.

TABLE III

A Comparison of the Effects of the Volume of Inoculum and the Length of the Needle Used to Inoculate Neutral Serum-Virus Mixtures

⅞ inch needle				¼ inch needle			
Test tube No.	Monkey No.	Amount inoculated	Result	Test tube No.	Monkey No.	Amount inoculated	Result
		cc.				*cc.*	
1	1	1.0	Remained well	6	11	1.0	Dead, 6 days. Colitis. Cord Sections showed no evidence of poliomyelitis. India ink in subarachnoid space over the site of inoculation and over the spinal cord
	2	0.25	Paralyzed, 18 days. Ink found at site of inoculation and in lateral ventricle. None on surfaces of brain or cord		12	0.25	Remained well
2	3	1.0	Remained well	7	13	1.0	Remained well
	4	0.25	Remained well		14	0.25	Remained well
3	5	1.0	Dead next day of unknown cause. India ink at site of inoculation, and subarachnoid space above it	8	15	1.0	Remained well
	6	0.25	Paralyzed, 17 days. Ink found at site of inoculation and in lateral ventricle below it		16	0.25	Remained well
4	7	1.0	Paralyzed, 12 days. Ink found in subarachnoid space over both cerebral hemispheres, also at the base of the brain and throughout the spinal cord	9	17	1.0	Remained well
	8	0.25	Paralyzed, 9 days. Ink confined to site of inoculation only **		18	0.25	Remained well
5	9	1.0	Remained well	10	19	1.0	Remained well
	10	0.25	Remained well		20	0.25	Remained well
Controls *		1.0	Paralyzed, 4 days. Ink in all the ventricles, base of the brain and spinal cord	Controls *		1.0	Paralyzed, 10 days. Ink in the subarachnoid space, base of the brain and site of inoculation
		0.25	Paralyzed, 5 days. Ink at site of inoculation and lateral ventricle			0.25	Paralyzed, 10 days. Ink at site of inoculation only **

* The controls received a similar mixture of normal monkey serum, 5 per cent virus filtrate and India ink.

** These animals had been inoculated 2 months previously with serum-virus mixtures and survived the tests. The India ink was confined mostly to the cavitation of the sterile brain abscesses often seen in monkeys examined a few weeks after intracerebral injection (see Fig. 22).

Experiment IV

To determine whether or not direct admixture with the cerebrospinal fluid would prove this point, a group of 5 monkeys was injected with 1 cc. of mixtures of virus and serum, prepared as described above, but without India ink. The inoculations were made below the dura and into the subarachnoid space above the right cerebral hemisphere. This was accomplished by surgical trephining of the frontal bone at the usual site of inoculation. The area exposed was made large enough so that sufficient assurance could be had that the brain substance was not touched upon subdural insertion of the needle. All of these animals remained well during an observation period of 2 months, whereas a group of 4 controls inoculated similarly, but receiving a mixture of the virus and normal monkey serum, all developed poliomyelitis within the usual incubation period.

Of another group of 4 monkeys, each inoculated with 1 cc. into the cisterna magna, none showed evidence of infection while the four controls became paralyzed.

Experiment V

Since during the process of an intracerebral inoculation the brain is traumatized, we decided to investigate the combined effect of direct inoculation into the spinal fluid and simultaneous brain trauma. Accordingly, a group of 12 monkeys was inoculated with 1 cc. of a neutral serum-virus mixture (4 cc. of undiluted pooled human convalescent serum and 4 cc. of 5 per cent virus suspension) intracisternally. In 6 of these monkeys, immediately following inoculation, a sterile 7/8 inch needle was pushed into the brain at the usual site of inoculation and then withdrawn. The other 6 monkeys were treated in a similar manner with a 1/4 inch needle. None of the 12 animals developed poliomyelitis, while the control in each group became paralyzed.

Experiment VI

Having no indication from the above experiments that the dilution phenomenon took place *in vivo*, we attempted to determine whether it would occur *in vitro*. Therefore, monkeys were injected from 3 tubes containing mixtures which consisted of equal quantities (4 cc.) of 5 per cent virus filtrate and human con-

valescent serum, 0.5 cc. of which was diluted, after the usual incubation time, with 2 cc. of normal monkey spinal fluid, giving a dilution ratio of 1:5. Twelve monkeys, 4 injected from each tube, received intracerebrally 1 cc. each of these mixtures prior to dilution with the spinal fluid, and 6 monkeys, 2 injected from each tube, received 1 cc. each of the mixtures following dilution. None of these animals developed the disease. Two controls receiving the virus and normal monkey serum and 2 others injected with virus, normal monkey serum and spinal fluid, all became infected.

Discussion

Our experiments indicate that the manner in which material, following intracerebral inoculation, is distributed, resembles in many respects that noted after intrathecal inoculation, as described by Hurst.[6] While certain variations in the course and extent of the diffusion were observed, some admixture of the material with the cerebrospinal fluid occurred in almost every instance. Generally, little of the material was found in the brain substance at the site of inoculation, except in monkeys that had received intracerebral inoculations in other experiments, in which case most of the ink was usually confined in the necrotic area of the previous site of inoculation. When larger amounts or longer needles were used, it appeared that the material more readily found its way into the cerebrospinal fluid. It may be stated, however, that the results are uncertain in so far as the final deposition of the material is concerned, but, in any event, some seepage into the cerebrospinal fluid is to be expected.

What, if any, correlation exists between the diffusion of material and its ultimate effect upon the infectivity of the neutral virus-serum mixtures cannot be answered from the data at hand. From the results of Experiment III, it appears that intracerebral inoculations with a ¼ inch needle tend towards greater regularity, since monkeys receiving the same mixtures were infected when the ⅞ inch needle was used. At first, we were inclined to attribute the consistent infectivity of the neutral mixtures inoculated with a short needle to the fact that less seepage might have taken place. However, admixture with the cerebrospinal fluid seems to have no bearing upon the infectivity of these mixtures since, as observed in Experiments IV and V, direct subarachnoid or cisternal introduc-

tion or intracisternal inoculation with simultaneous brain trauma caused no reactivation of the inactive mixtures.

We sought an explanation for irregularities on the ground that the dilution phenomenon may occur. Although this has been reported to take place with a mixture of immune serum and poliomyelitis virus by Schultz and his collaborators,[11] we were unable to demonstrate, in our experiments, that the dilution phenomenon ensued either *in vitro* or within the animal body.

It is, therefore, difficult to state what factors may account for unexpected infections when a presumably neutral mixture of serum and virus is injected into a group of monkeys. So far as this work has been carried, there is some indication that the use of a ¼ inch needle or direct intracisternal inoculation may be an improvement over the usual intracerebral method for performing neutralization tests, but further studies on a larger scale are necessary to verify this.

Summary

1. The distribution of material throughout the central nervous system of *Macacus rhesus* monkeys, subsequent to intracerebral inoculation, was studied by the injection of India ink. The experiments indicated that certain variations in the degree and extent of diffusion occurred, but in most instances the inoculum rapidly entered the cerebrospinal fluid either via the subarachnoid space or ventricles. Except for the site of inoculation, no carbon particles were found in the brain substance itself. The India ink was deposited on the surfaces of the brain or cord.

2. It appeared that when material was inoculated in larger amounts or with longer needles, it more readily entered the cerebrospinal fluid.

3. In an experiment where apparently neutral mixtures of poliomyelitis virus and immune serum were inoculated intracerebrally into monkeys with ¼ inch needles and ⅞ inch needles, none of the 10 monkeys injected with the shorter needles developed poliomyelitis, while 4 of 10 monkeys receiving the mixtures with ⅞ inch needles succumbed to the disease.

4. Direct inoculation of serum-virus mixtures into the subarachnoid space or cisterna magna did not render these neutral mixtures active, nor were these mixtures infective when inoculated

intracisternally accompanied by brain trauma, although controls similarly inoculated were uniformly infected.

5. Experiments devised to demonstrate the occurrence of the dilution phenomenon *in vivo* or *in vitro* were negative.

The authors gratefully acknowledge the technical assistance given by Mr. Angelo Campagna.

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DESCRIPTION OF PLATES

PLATE 45

FIG. 1. Dorsal view of the brain of Monkey No. 21. India ink is deposited on the surface of the frontal lobe.

FIG. 2. Ventral view of the brain of Monkey No. 21.

FIG. 3. Monkey No. 300 with skull exposed showing external leakage following injection. India ink is deposited in the area surrounding the trephined opening.

FIG. 4. Ventral view of brain and spinal cord of Monkey No. 300. India ink is deposited over the surfaces of the brain, cerebellum and spinal cord.

FIG. 5. Dorsal view of brain and spinal cord of Monkey No. 300 with section through the site of inoculation showing India ink deposited in the lateral ventricle and on the surfaces of the brain and spinal cord.

FIG. 6. Brain of Monkey No. 300 sectioned through another plane at the site of inoculation. Note the path of the needle and ink in the lateral ventricle.

FIG. 7. Brain of Monkey No. 180 sectioned through the site of inoculation. The India ink is confined to the necrotic cavity present as a result of an inoculation given 2 months previously.

FIG. 8. Dorsal view of brain and spinal cord of Monkey No. 88. The surfaces are heavily coated with India ink.

FIG. 9. Ventral view of the brain and spinal cord of Monkey No. 88 showing considerable deposit of India ink.

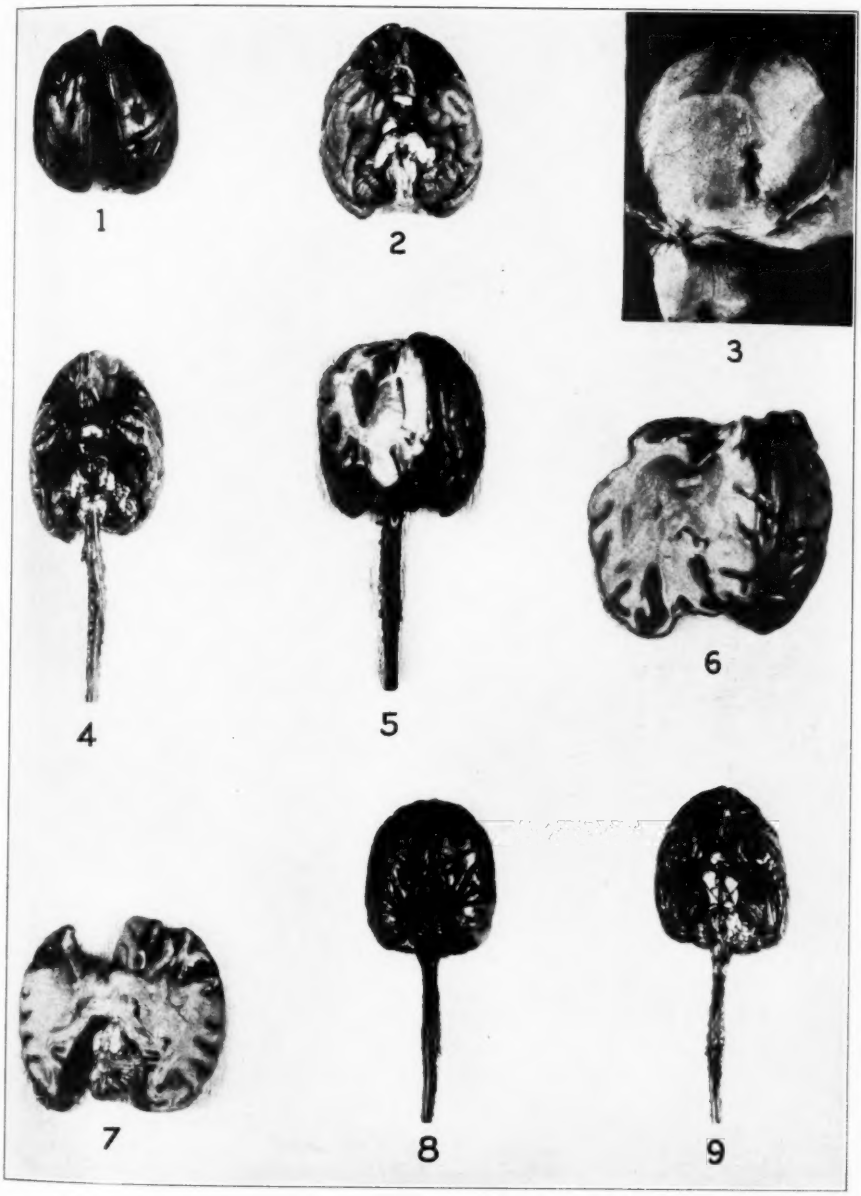

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PLATE 46

FIG. 10. Dorsal view of brain of Monkey No. 295. India ink is deposited on the entire surface of the right frontal lobe up to the fissure centralis.

FIG. 11. Sagittal section through the right hemisphere at the site of inoculation of the brain of Monkey No. 295. The area into which the inoculum has been deposited is distended.

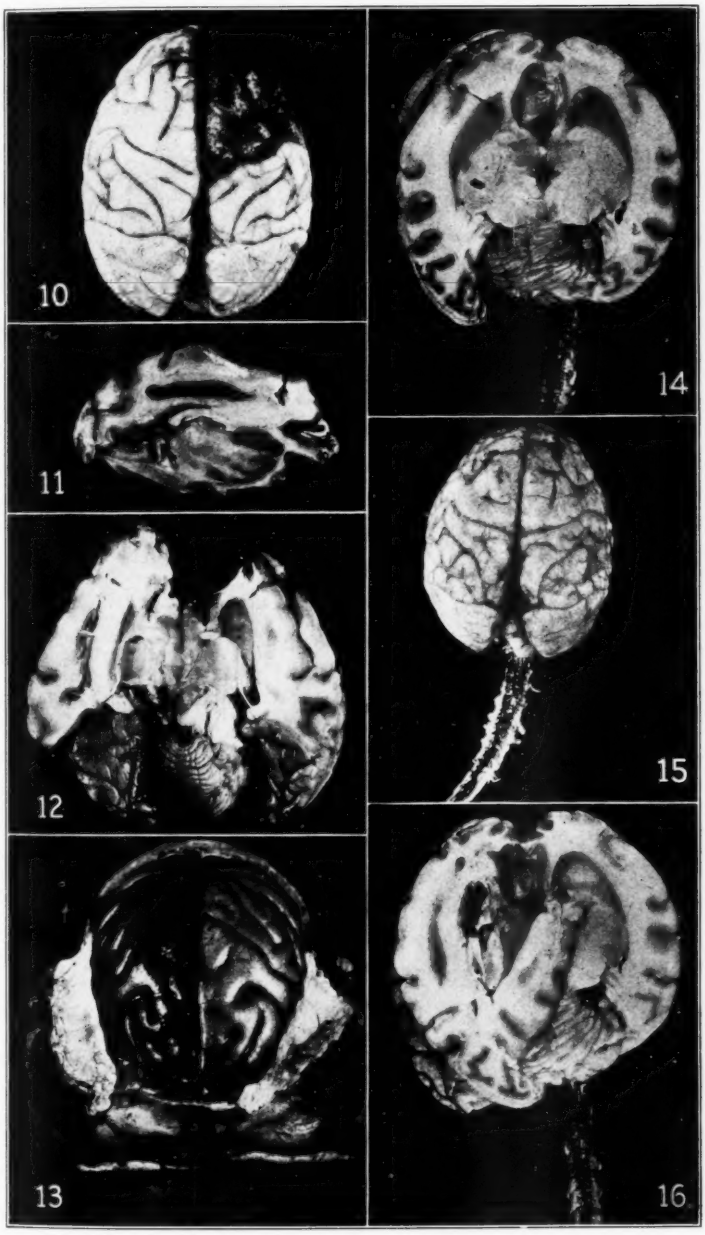
FIG. 12. Brain of Monkey No. 137 sectioned through the site of inoculation. Note the India ink on the surface and in the area inoculated. Distention is also present here but to a lesser degree than that noted in Monkey No. 295.

FIG. 13. Dorsal view of the brain of Monkey No. 225 *in situ* with dura removed showing deposit of India ink over the entire surface of the cerebral hemisphere opposite the side inoculated.

FIG. 14. Brain of Monkey No. 225 sectioned through the site of inoculation. India ink is present in the ventricles and on the spinal cord. The path of the needle is made clearly visible by the deposit of India ink.

FIG. 15. Brain and spinal cord of Monkey No. 207. No India ink is evident on the surface of the brain but it is abundant on the spinal cord.

FIG. 16. Brain of Monkey No. 207 sectioned through the site of inoculation. A large amount of India ink is seen in the lateral ventricle and on the spinal cord.



Schaeffer and Muckenfuss Experimental Poliomyelitis

A MODIFICATION OF THE MASSON TRICHROME TECHNIQUE FOR ROUTINE LABORATORY PURPOSES *

JACQUES GOLDNER, M.D.

(From the Department of Surgical Pathology of the New York Hospital and Cornell University College of Medicine, New York, N. Y.)

Trichrome methods are rapidly replacing the ancient hematoxylin-eosin technique so largely used in pathology. With their use diagnosis becomes easier as a result of the topographical delimitation of the connective tissue, and the reactions of this tissue itself, such as sclerosis and the like, become at once apparent. There is no lack of good trichrome techniques in our armamentarium, but for rapid results the routine worker can but very rarely have recourse to such stains as Mallory's excellent phosphotungstic acid hematoxylin method, which necessitates treatment with iodine and bleaching with sodium thiosulphate, and a subsequent oxidation and reduction with potassium permanganate and oxalic acid. Furthermore, sections so treated only begin to show a rich color and good detail after some 6 to 12 hours of staining.

Domagk has devised a striking modification of Mallory's connective tissue stain (at the laboratories of the I. G. Farbenindustrie in Eberfeld-Leverkusen) by replacing fuchsin with a stable and exclusively nuclear stain (Kernechtrot) which is a very brilliant carmine. One does not, however, obtain the full tinctorial effects on the nuclei for some 30 minutes and, often enough, differentiation is found to be necessary. The method, excellent in itself, cannot conveniently be utilized in laboratories where there is much routine work and a need for the rapid mass production of sections. There are other modifications of the Mallory trichrome method — the galleïn-orange-aniline blue and the acid alizarin blue (proposed by Petersen [1]) among them, but they are unsuited to rapid work.

Masson's [2, 3] trichrome method remains one of the best, combining as it does the most precise of hematoxylins (Heidenhain's iron hematoxylin) with a reliable cytoplasmic stain that gives a wealth of detail (acid fuchsin with ponceau de xylidine), and a very selective stain for connective tissue (light green or aniline blue). This

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method requires mordanting with ammonio-ferric alum for 24 hours, followed by staining with Regaud's hematoxylin (the formula based on Heidenhain's) for a similar length of time. One may shorten the process to an hour by mordanting for 30 minutes and staining a like period at 50° C. If one shortens the process still further, however — say to 5 minutes in each solution — the stain becomes neither precise nor stable; the nuclei soon fade out, even during the finishing of the sections, being replaced by fuchsin. Furthermore, they must be differentiated in picric acid alcohol and this process should be followed under the microscope to avoid over-differentiation. When one must be staining a hundred sections at a time, this very excellent trichrome method becomes difficult of application. Although fine in itself, it was not devised for busy pathologists.

I have, therefore, modified it so that it might be made more applicable to routine needs. The Heidenhain-Regaud hematoxylin has been replaced by the ferric trioxyhemateïn of Hansen; the other elements of Masson's stain have been diluted to avoid areas of concentration and to give more transparency to the sections, as well as obviating the evils of long exposure to a stain. Phosphomolybdic acid has been replaced by phosphotungstic and the strength of this increased to 5 per cent in order to shorten the time element. Finally, a third cytoplasmic stain (orange G) has been added to accentuate the erythrocytes, to increase the yellow tones and to afford a supplementary color. The colloid of the thyroid, for instance, may take on a green color, or various shades of red with the fuchsin, or it may come out a pure orange. This probably depends upon varying chemical characteristics of the colloid under varying metabolic circumstances. With this modification one thus obtains a good stain that resembles Masson's, without always showing all of its characteristics, completed within a period of from 20–40 minutes. Naturally, the time required for deparaffinization, and so on, is not reckoned in. In appropriate cases the procedure may be shortened to even 10 or 12 minutes.

Hansen's ferric trioxyhemateïn is a stable precise dye of the color of lithographic ink, sometimes verging on the sepia. Of itself, it gives a wealth of detail (granules, cilia, fibrillary structures, cuticulae of epithelium, and even spirochetes). It does not obscure the connective tissue, particularly after formalin fixation, where

Heidenhain's hematoxylin produces grayish areas that cannot be decolorized. It may be differentiated, if needs be, in weakly acidulated water or alcohol, but this is not usually necessary, the various tissue elements appearing in gradations of gray to black through the brick red background of the fuchsin-ponceau, provided that this has been sufficiently diluted. In short, it may be rendered a purely nuclear stain by the addition of sulphuric acid to the trioxyhemateïn solution. In this way one need not fear overstaining which is important in laboratories where one has insufficient leisure to dedicate much time to individual sections.

It is for the same reason that the cytoplasmic stains of Masson's method have been diluted. The tinctorial effects are obtained in a minimal period of time, the dilution prevents overstaining and affords acceleration of the process; finally, the sections show increased transparency and have more agreeable tonalities, both under the microscope and on the projection screen. This variant may be used on any material fixed in any of the usual fluids, provided one observes the usual formalities — iodine and sodium thiosulphate after Zenker's fixative, or alcohol and lithium carbonate after Bouin's, and so on. It may be used on paraffin sections from tissue fixed in neutral formalin or alcohol-formalin, but the orange G tends to stain a little more irregularly and diffusely in these than in those fixed in Zenker's or Bouin's fluids.

This method has been adopted as the routine stain in the laboratories of the Department of Surgical Pathology of the New York Hospital and Cornell University Medical College. It has proved to be a vast improvement over the trichrome light green method used with success for the past 5 years. Its advantages are: (1) increased transparency with consequently increased histological detail, particularly shown in muscular tissue; (2) much more precise nuclear detail and no replacement of the nuclear stain by the red elements; details of mitotic figures are beautifully brought out; (3) no "piling up" of the light green in connective tissue or mucus, hence no obscuring of connective tissue details; and (4) better color values for the purposes of microphotography.

Method of Procedure

1. Stain deparaffinized sections taken out of water in Hansen's iron hematoxylin for 1–5 minutes. The dye may be used pure, or it

may be acidified with 2 parts of 2 per cent aqueous sulphuric acid to 8 parts of the dye. Longer staining does no harm, as it can not overstain the tissue if it has been acidulated. The solution may be used repeatedly, but it must be filtered before using. It must be sepia black; if it becomes greenish a new supply should be made up. It keeps for approximately 6 weeks in ordinary use.

2. Wash the sections at the tap as long as yellowish brown clouds come off in the water. After 5 minutes the nuclei should be a rich black.

3. Stain in Masson's fuchsin-ponceau mixture, diluted 10 times with water acidulated to 0.2 per cent (1:500) with acetic acid, for 5 minutes or more.

4. Rinse in distilled water acidulated in the same fashion. If the city supply be not too alkaline, tap water may be used. Usually a few drops of glacial acetic acid in tap water will work well.

5. Treat for 15 seconds to 30 minutes with phosphotungstic acid orange G. A few minutes usually suffice amply.

6. Repeat the rinsing as in Step 4.

7. Stain for 5 minutes in Masson's light green solution diluted 10 times with water acidulated as above.

8. Rinse as in Step 4 for 5 minutes to eliminate the phosphotungstic acid and to differentiate the various color tones.

9. Dehydrate in the usual manner with ascending percentages of alcohol, clear in xylol and mount in balsam.

Results: The nuclei are black to brownish black; the cytoplasm is brick red (certain granules stain more golden); erythrocytes are yellowish vermilion to orange; collagen and mucus bluish green. Other structures appear in various shades of gray superimposed upon the reddish background.

Variants

(A) If time is no object, one may prolong the hematoxylin stain to 15 minutes, differentiate in 2 per cent aqueous sulphuric acid until one obtains the desired accents on elements to be brought out, and then wash in water and proceed with the rest of the stain.

(B) Fuchsin-ponceau may be replaced by azophloxine (Hollborn), which resembles eosin but does not "pile up" or overstain. Its tones are warmer and the erythrocytes are very selectively demonstrated in shades of cardinal red to salmon pink. Further-

more, it is extremely stable and has been exposed to sunlight during an entire summer without deterioration. A 0.05–0.1 per cent solution is prepared in water containing 2 drops of acetic acid to 100 cc. of water. The solution should have thymol, or other disinfectants added to it to prevent the formation of molds. It stains almost instantaneously, but sections may be left in it for 2–5 minutes. Sections stained with this dye remind one of those prepared by Prenant's trichrome method which was, in the days before Masson's method appeared, one of the most employed techniques in French laboratories, or those under French influence.

Formulas and Preparation of Staining Solutions

1. Hansen's Trioxyhematein

Solution A: Dissolve 10 gm. of ammonio-ferric alum (amethyst crystals) and 1.4 gm. ammonium sulphate in 150 cc. of distilled water, warming gently. This may also be done in the cold.

Solution B: Dissolve 1.6 gm. of hematoxylin in 75 cc. of distilled water in a porcelain dish over the flame.

When the two solutions have thoroughly cooled, pour *Solution A* into *Solution B* (never *vice versa!*); the mixture, at first brown, changes to blue and then to deep violet. The color changes may be checked up by placing drops of the mixture on filter paper from time to time. While pouring A into B the containers should be constantly agitated to ensure even mixing. When the color has become violet heat the mixture cautiously to avoid overoxidation and do not wait for the boiling point to be reached if the test drops on the filter paper are a brownish black, or sepia color. Under no circumstances boil for more than 30–60 seconds. Chill the mixture abruptly, after the end-point of the reaction has been reached, by floating the dish on cold water. When bottling, fill up to the neck to avoid leaving an air space at the top that might cause superoxidation. Bottles of Pyrex, or some such glass, are better than those of ordinary glass, which tends to be alkaline. The final solution should be sepia black; if olive green it will stain poorly. The greenish tinge indicates overoxidation. The solution may be restored to its original sepia color by adding 10 per cent oxalic acid, drop by drop, until the desired tone is obtained. The solution keeps for a long time. In order to render the stain strictly and exclusively

nuclear, 2–4 parts of 1 per cent aqueous sulphuric acid should be added to 8 parts of the dye.

2. Masson's Fuchsin-Ponceau (Dilute Formula)

Ponceau de xylidine (Krall or Hollborn)	0.2 gm.
Acid fuchsin (acid rubin) (any good brand)	0.1 gm.
Distilled water with 0.2 per cent acetic acid	300 cc.

If the original formula is kept in stock, it should simply be diluted 10 times.

3. Phosphotungstic Acid Orange G

Phosphotungstic acid	3–5 gm.
Orange G (Hollborn "standardized")	2 gm.
Distilled water	100 cc.

4. Light Green

Light green (Lichtgrün)	0.1–0.2 gm.
Distilled water with 0.2 per cent acetic acid	100 cc.

NOTE: Since submitting this manuscript, several thousand sections have been stained in our laboratory. During this time we have perfected the method and simplified it still further.

Weigert's iron hematoxylin nuclear stain gives fine results, often better than those obtained with Hansen's hematoxylin, and its preparation is far simpler. In order to avoid the necessity for differentiating with acid, the dye should be diluted with an equal volume of 50 per cent alcohol, as in Petersen's method. With this there is no overstaining within 5–10 minutes. After staining wash the slides with 50 per cent alcohol acidulated with 0.1 per cent hydrochloric acid. It is well to increase the amount of hematoxylin in the formula from 1 to 1.25 gm. in 100 cc. of alcohol. The mixture of hydrochloric acid and ferric chloride, as given in the formula, remains unchanged. The stain will keep for 1 day only and must therefore be made up fresh each day. Harris' hematoxylin, if overstained slightly (5–10 minutes), gives excellent results and it is not necessary to differentiate the stain as the acids that are used in the other stains later extract a certain amount.

Doctor Foot has found it advisable to add the orange G to the mixture of ponceau and acid fuchsin, in which case the sections may be mordanted in phosphotungstic acid with or without the addition of orange G. This dye is taken by the erythrocytes, keratin, sheaths of peripheral nerves (neurokeratin?) and the mature colloid of the thyroid, which transforms the stain from a trichrome to a tetrachrome. Following Zenker's and Bouin's fixatives, Hollborn's orange G is preferable to "certified"; following the alcohol-formalin fixative the reverse is true. The cytoplasmic stain, in this case, is made up as follows:

Ponceau-acid fuchsin, stock mixture	10 cc.
Orange G, 2 per cent aqueous solution	10 cc.
Distilled water	80 cc.

Personally I prefer the cytoplasmic stain in which the ponceau is reinforced with azophloxine to demonstrate eosinophilic granules and erythrocytes. The formula is as follows:

Masson's Ponceau-Fuchsin Stock Solution

Ponceau de xylidine, 1 per cent aqueous solution	3 parts
Acid fuchsin, 1 per cent aqueous solution	1 part

Staining Solution

Above mixture	5–10 cc.
Azophloxine, 0.5 per cent aqueous solution	2 cc.
Distilled water, acetified	88 cc.

The orange, in this instance, is introduced into the stain through the phosphotungstic acid bath.

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USEFUL METHODS FOR THE ROUTINE EXAMINATION OF BRAIN TUMORS *

NATHAN CHANDLER FOOT, M.D.

(From the Department of Surgical Pathology of the New York Hospital and Cornell University Medical College, New York, N. Y.)

The diagnosis of brain tumors has become increasingly complex with the added complexity incidental to the more accurate classifications resulting from the work of the Spanish school along these lines. Where "glioma" formerly sufficed for a diagnosis of neuroglial neoplasms, we must now distinguish between tumors arising from spongioblasts, astrocytes, oligodendroglial and ependymal cells. In routine work the making and impregnating of frozen sections is irksome, unless one be busied with little else. The technician must be highly trained and capable of using discrimination; otherwise the pathologist is constrained to do his own technique. In a laboratory where other routine material is coming through uninterruptedly this is often, if not usually, difficult of accomplishment.

It is the purpose of this article to set forth the routine method adopted in this laboratory, whereby any competent technician may prepare paraffin sections for the diagnosis of brain tumors along with the daily routine. It has been in use for about five years now and has been very satisfactory. Our diagnoses, checked against occasional autopsy diagnoses of our neuropathologist, have been in perfect agreement with his, which were made on frozen sections impregnated by the del Río Hortega method. Our procedure is as follows:

FIXATION

It is important to employ a number of fixatives when dealing with brain tumors; if one does not give good results, another will, so that three or four should be used, depending upon the amount of material to be fixed. If the specimen be very small, one should choose that fixative most likely to succeed.

Formalin-Alcohol: This is the routine fixative for the daily work in our laboratory. It consists of 10 parts of strong formalin to 90 parts of 95 per cent alcohol. Blocks of tissue not thicker than 4 mm. are placed in this solution for 7 to 8 hours. It removes a

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good deal of the lipins, clears the background and gives excellent results. Aqueous neutral 10 per cent formalin is not to be recommended for these purposes.

Bouin's Fluid: This is used in the formula recommended by Masson: water 30 parts, strong neutral formalin 10 parts, 2 per cent trichloracetic acid (instead of glacial acetic) 2 parts, and picric acid to saturation. Thin blocks of tissue are fixed in this for 12 to 24 hours.

After fixation the tissues should be washed in water and then treated for at least 2 hours with 80 per cent alcohol saturated with lithium carbonate.

Cajal's Formalin-Ammonium Bromide: This is made up of strong formalin 15 parts, ammonium bromide 3 parts, and water to make 100 parts. It is the classic fixing fluid for tissues to be impregnated by the various silver and gold methods, but it is not generally realized that it gives superior results in trichrome sections as well.

Zenker's Fluid: The regular formula, which need not be given here, is used. Sections from Zenker-fixed tissue should be treated with weak alcoholic iodine for a few minutes, and then with a 0.5 per cent aqueous solution of sodium thiosulphate, in the usual manner, to remove the mercury precipitate before staining.

In practice, the first three methods of fixation usually suffice; the last may be held in reserve if Zenker-fixed material is needed later on.

Staining Method

The Goldner modification [1] of the Masson trichrome technique has become our routine stain and it gives excellent results in the case of brain tumors which, in most instances, may be diagnosed through the use of this method alone, without recourse to silver impregnations, although the latter are always used for purposes of confirmation. Tissue fixed in the fluids just enumerated is embedded in paraffin and cut in the usual manner.

Staining Solutions

1. Hansen's Trioxyhematein

Dissolve 10 gm. of ammonio-ferric alum (amethyst crystals) and 1.4 gm. of ammonium sulphate in 150 cc. of distilled water,

warming gently over a flame. Next 1.6 gm. of hematoxylin are dissolved in 75 cc. of distilled water in a porcelain dish with a capacity of 250 cc. or more, over a flame. When the two solutions have cooled, they are mixed by pouring the former into the latter (never the reverse!), the container being agitated continuously to ensure perfect mixture. The color changes from brown to blue and then to violet. When the violet stage has been reached, the mixture is cautiously heated until test drops on filter paper are sepia black. Never boil for over 1 minute and do not wait for the mixture to boil if the color is arrived at before the boiling point is attained. Cool the mixture suddenly by floating the dish in cold water, to prevent overoxidation. A greenish tinge indicates this. The addition of 10 per cent oxalic acid, added drop by drop, will restore the desired color by reduction. The stock solution keeps a long time, but should be tightly stoppered to avoid oxidation. In order to render the stain selectively nuclear, 2 to 4 parts of 1 per cent aqueous sulphuric acid should be added to 8 parts of the stock solution.

(*1 a.*) Harris' hematoxylin made according to the usual formula may be substituted for iron hematoxylin. It works equally well, but the sections should be overstained (say 5 minutes), as the picric acid and acetic acid used subsequently tend to take some of it out of the tissue.

2. *Masson's Fuchsin-Ponceau, Modified with Orange G (Dilute Formula)*

To 300 cc. of distilled water acidulated with 0.2 per cent acetic acid, 0.2 gm. of ponceau de xylidine (Krall) or xylidine ponceau (Hollborn), and 0.1 gm. of any good brand of acid fuchsin, are added. We have found it advisable to shift the orange G from the phosphotungstic acid bath (as proposed by Goldner) to this solution, adding 0.2 gm. of the dye. It is more convenient to make up a stock solution of the original strength (2, 1, and 2 gm.) and dilute it 10 times with acetified water, as the weighing of tenths of a gram is troublesome.

3. *Phosphotungstic Acid*

This replaces the original phosphomolybdic acid in Masson's formula. It should be made up in 3–5 per cent strength.

4. Light Green

This is a 0.1 per cent solution of light green (Lichtgrün) in acetified distilled water (0.2 per cent acetic acid).

Procedure

Sections are stained in the iron hematoxylin for from 1–5 minutes, in Harris' solution for 5 minutes. With sulphuric acid added to the iron hematoxylin, it cannot overstain and may be used repeatedly, filtering frequently, until it becomes greenish, when it should be replaced. The sections are then washed at the tap until yellowish brown clouds of dye no longer come away in the case of iron hematoxylin, or until the sections are blue in that of Harris' hematoxylin.

The sections are next stained in the fuchsin-ponceau-orange G mixture for 5 minutes or longer and are then rinsed in acetified water. If the city supply be not alkaline, tap water may be used.

The slides are next immersed in the phosphotungstic acid solution for a few minutes and rinsed in acetified water, after which they are stained in the light green solution for 5 minutes and treated with acetified water for a like period to eliminate any remaining phosphotungstic acid and to differentiate the color tones. They are then dehydrated and mounted in balsam in the usual manner.

The procedure is really not at all formidable and the technician soon becomes accustomed to its different steps.

Silver Impregnation

Solutions

1. *Pyridine-Glycerin:* This is 2 parts of pyridine to 1 part of glycerin.

2. *Impregnating Fluid:* Into 10 cc. of a 10.2 per cent silver nitrate solution, strong ammonia is dropped from a dropping-bottle until the resulting precipitate is almost dissolved; the process should best not be carried to the "water-clear" stage, but the solution should be slightly turbid. To this, 10 cc. of 3.1 per cent solution of sodium carbonate in distilled water is added and the mixture made up to 100 cc. with distilled water.

3. *Reducer or Developer:* This is made up of 3 cc. of 1 per cent

sodium carbonate (buffer) in distilled water, 1 cc. of strong formalin, and distilled water to make 100 cc.

4. *Toning Solution:* This is a 1:500 solution of gold chloride in distilled water.

5. *Intensifier:* Oxalic acid 2 gm., strong formalin 1 cc. and water to make 100 cc.

6. *Fixing Fluid:* A 5 per cent solution of sodium thiosulphate, or "hypo."

7. *Counterstain (van Gieson):* To 10 cc. of a 1 per cent solution of acid fuchsin add 90 cc. of a solution of picric acid in water, saturated at room temperature.

Procedure

1. Sections are deparaffinized and carried into pyridine-glycerin for 24 hours. (Keep under hood to avoid unpleasant odors and possible headaches.)

2. Rinse in 95 per cent alcohol followed by distilled water.

3. Impregnate in the silver solution for 2½ hours at 40° C. (If the solution is cold at the start, allow 15 minutes for it to warm up, making the time 2¾ hours.)

4. The sections are next washed in distilled water.

5. Reduce for 5 minutes in the developer, until the sections turn dark brown.

6. Wash in tap water.

7. Next tone for 5 minutes in the gold chloride solution.

8. A wash at the tap follows.

9. Intensify in the intensifier for 5 minutes.

10. Rinse in tap water.

11. Fix in the sodium thiosulphate solution to remove surplus unreduced metal.

12. Wash again in tap water.

13. The sections may then be counterstained in the van Gieson solution, but this is optional.

14. Dehydrate and clear sections and mount in balsam.

Here again, the rather formidable schedule proves to be less so as one becomes used to it. The silver solution may be made up in bulk and kept in a dark ice-box for use as needed. If a precipitate forms it can usually be redissolved by gentle shaking, or it may be filtered out.

Modified Ramon y Cajal Method

It often happens that one would like to ascertain something concerning the nervous tissue proper in connection with the infiltration and invasion of brain tumors. After experimenting with several methods we have found that a modified block impregnation, as originated by Ramon y Cajal, gives the best results.

Procedure

Blocks of tissue not over 4 mm. in thickness are fixed in 25 per cent aqueous chloral hydrate for 24–48 hours, the latter time being preferable. This method requires this special fixation, which should be noted. The blocks are then rinsed in distilled water for a few seconds and blotted with filter paper to remove the excess water. They are next transferred to a mixture of 95 per cent alcohol and 4 drops of ammonia for 24 hours, after which they are rinsed for a few minutes in distilled water.

Impregnate with a 1.5 per cent aqueous silver nitrate solution at 37° to 38° C. for a week in the dark. If the solution becomes yellow, as it may after 30 to 60 minutes, it should be renewed with fresh solution, after which it will remain colorless.

The blocks are then developed with a mixture of 2 gm. of pyrogallic acid, 8 cc. strong formalin, and 100 cc. of distilled water for 12 hours, or at least overnight. Then wash for 3 to 4 hours in distilled water and transfer to 80 per cent alcohol. The alcohol will turn yellow but this has no significance. The blocks are then carried through whatever fluids one may desire for paraffin embedding. Sections cut from the paraffin blocks need no further treatment save deparaffinization; it is well to discard the first few sections cut, as the periphery of the blocks is usually over-impregnated.*

Results

By preparing sets of sections from tissue fixed in alcohol-formalin, staining one with the Masson-Goldner ** trichrome

* We are indebted to Dr. José Nonidez of our Department of Anatomy for calling to our attention this excellent method.

** If Harris' hematoxylin has been used, the nuclei will stain the usual purplish color. With orange G added to the ponceau-fuchsin, we find that the orange dye is specifically selective for myelinated nerve sheaths which, in this case, take no other color but come out bright orange-yellow. Fibrin shows more affinity for the

method, and impregnating the other with silver, one may have a reasonably accurate diagnosis in a few days time. The sections from material otherwise fixed will then be ready in a day or two and may be used for checking up on this diagnosis. The Ramon y Cajal sections will, of necessity, not be ready for a couple of weeks. It so happens that formalin-alcohol is one of the most satisfactory fixatives, so that the early sections are often just as good as those that follow later.

Masson-Goldner Sections: Brain tumors stained by this method show sepia to black nuclei, pink neuroglia, red cytoplasm and cell processes, green connective tissue about the vessels and meninges, and coral red to orange erythrocytes. Medullated and non-medullated nerve fibrils stain more densely red than the neuroglia and may even come out a vermilion color. Sections from tissue fixed in aqueous formalin (which is not recommended) may show a greenish coloration of the neuroglia, which is misleading. In edematous tumors the coagulated serum sometimes stains a greenish hue. Fibrin stains brilliant vermilion to orange. The effects obtained with this stain are excellent and bring out the finer histological details of the astrocytes and other cells. It should be repeated that formalin-ammonium bromide gives very fine results with this trichrome method.

Silver Sections: It is not claimed that this will succeed with normal brain tissue, for it is not particularly satisfactory in demonstrating normal astrocytes, and so on. On the other hand, the less mature cells of tumors, even those of well differentiated astrocytomas and oligodendrogliomas, are well brought out; their processes are dark purple or brown, the nuclei are black, and the cytoplasm varies from warm brown to black or purplish. Vascular reticulum is usually black, but the impregnation is not directed toward the demonstration of this tissue, which is better shown in sections that have been treated with potassium permanganate and oxalic acid instead of pyridine-glycerin.

As has been said, the sections from blocks fixed in a certain fluid may not be very fine, but in this case those from one of the others will be quite satisfactory; often all of them come out equally well.

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ponceau. The orange G also stains keratin selectively and shares in the staining of muscle and erythrocytes with the red dyes, in the case of sections made from tissues other than nervous tissue.

With a neutral alcohol-formalin, formalin-ammonium bromide (which suppresses the impregnation of reticulum) and an acid Bouin's fixative one is practically certain of getting sections that will make accurate diagnosis possible.

The figures in the plate demonstrate results of silver impregnations on various tumors (Figs. 1, 2 and 3).

Ramon y Cajal Sections: These come out in the old gold and black color scheme familiar to those who have used Levaditi's method. The black, however, is confined to the neuraxons. The nuclei are deep brown. The collagen and reticulum do not stand out very prominently. This is of great importance, for there can then be no confusion between reticulum and nerve fibers.

The general effects are illustrated in Figure 4, which shows surviving neurons in a brain infiltrated by glioblastoma multiforme.

Discussion

We have thus far diagnosed astrocytomas, polar spongioblastomas, glioblastomas of the multiform type, oligodendrogliomas and a glioblastoma of sympathetic origin arising from the suprarenal region. This last tumor would have passed as an unusual carcinoma, in fact it did until silver impregnations demonstrated the morphology of the multipolar astrocytes it contained. Their processes were practically unnoticeable in the hematoxylin-eosin sections, but better shown in the trichrome sections, which led us to try silver impregnation. In this they came out beautifully.

There is no reason why the combination of the methods set forth in this paper should not be equally good for ependymomas and those tumors of the more immature type — the so-called medulloblastomas and neuroepitheliomas. We have found them excellent in the case of meningiomas. Naturally, they are also applicable to tumors of peripheral nerves, where they give splendid results and afford a means for accurate diagnosis.

Note: Miss Anna Mary McDowell has been of great assistance in the working out of these methods in our laboratory.

REFERENCES

1. Goldner, Jacques. A modification of the Masson trichrome technique for routine laboratory purposes. *Am. J. Path.*, 1938, **14**, 237–243.

DESCRIPTION OF PLATE

PLATE 47

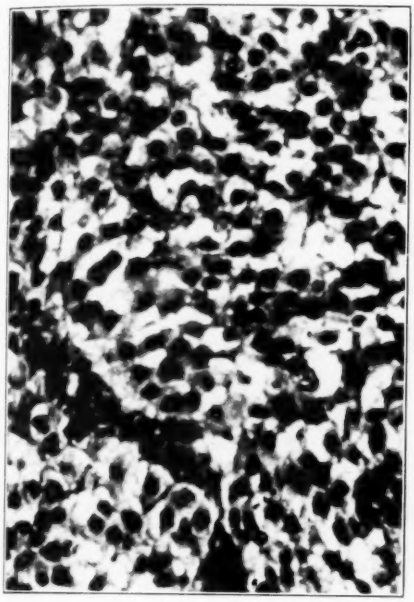
The photomicrographs were taken at a magnification of × 480, using a K 3 filter instead of the usual green filters employed with hematoxylin-eosin sections.

FIG. 1. A rather protoplasmic form of oligodendroglioma. Bouin fixation.

FIG. 2. A polar spongioblastoma. Alcohol-formalin fixation.

FIG. 3. A glioblastoma multiforme. Ramon y Cajal formalin-ammonium bromide fixation. These three figures are from sections impregnated by the pyridine silver method.

FIG. 4. A glioblastoma multiforme invading brain tissue, little of which has survived; only the neurofibrils have withstood the attack by the tumor cells. Chloral hydrate fixation.



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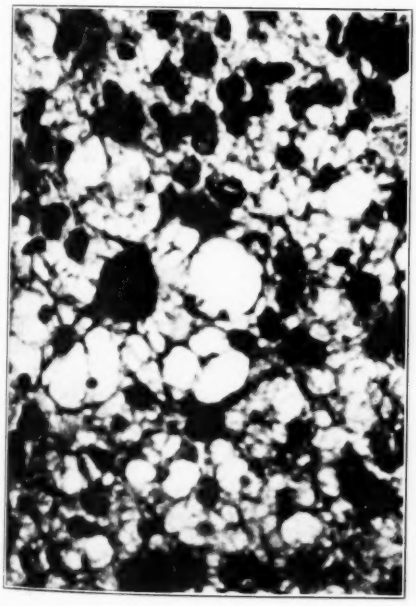

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Routine Examination of Brain Tumors